REGULATION OF PDGF CHAIN AND RECEPTOR EXPRESSION IN HUMAN MALIGNANT MESOTHELIOMA CELL LINES

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REGULATION OF PDGF CHAIN AND RECEPTOR EXPRESSION IN HUMAN MALIGNANT MESOTHELIOMA CELL LINES

Regulatie van PDGF keten en receptor expressie in humane maligne mesothelioomcellijnen

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CHAPTER 1

INTRODUCTION

1.1 GROWTH CONTROL, ONCOGENES AND TUMOUR SUPPRESSOR GENES

Growth control

In normal, untransformed cells, growth is a tightly regulated process. A single cell is capable of controlling its growth by integrating the input from both positive growth-stimulating and negative growth-inhibiting signals. Unrestrained cell proliferation is the hallmark of carcinogenesis and arises as a consequence of aberrations in this normal growth control. The importance of such disturbed growth-regulating mechanisms is underscored by the observation that *in vitro* tumour cells were found to proliferate independently of exogenous growth factors (1). The apparent ability to be able to grow without mitogenic factors supported the earlier suggestion of Temin (2) that growth of transformed cells may be caused by endogenous production of growth factors. This concept was later extended to the autocrine growth stimulation model (3).

It has become generally accepted that genetic damage is a central event in the process of loss of growth control. Disturbances in the growth-regulating mechanisms by changes in the genes that govern these processes, consequently enable cells to escape normal growth control. Genes, whose products contribute to uncontrolled growth, once they are genetically altered, can be classified as oncogenes and tumour suppressor genes. In the former category such genetic alterations give rise to a gain-of-function resulting in positive growth stimulation (e.g. by changes in growth factor-encoding genes), whereas in the latter growth-inhibiting properties are lost.

Oncogenes

The first clues about the identity of genes contributing to carcinogenesis have come from the studies on RNA tumour viruses. These viruses that induce tumours and transform cells in culture, contained sequences in their genomes that caused transformation of the host cell. These so-called viral oncogenes (*v-onc* genes) turned out to be highly homologous to cellular genes in vertebrates. The first example was provided by the normal cellular gene that was identified on the basis of its similarity to the transforming *src* oncogene of Rous sarcoma virus (4). Based on their homology to viral oncogenes, such normal cellular genes were therefore collectively termed cellular (proto)-oncogenes and it was suggested that these *c-onc* genes may contribute to tumour formation in a positive way by spatial or temporal deregulated expression (e.g. by amplification, translocation or point mutation).

Evidence for a role of these proto-oncogenes in human tumours came from gene transfer experiments, in which the transforming genes isolated from these tumours appeared to stem from normal cellular genes. In this way it was found that activation

and transformation by the H-*ras* proto-oncogene, which was isolated from a bladder carcinoma, were the consequence of a single point mutation in the coding sequence of this gene (5).

Both gene transfer experiments and analyses of transforming sequences from RNA viruses have revealed many different oncogenes that play a role in tumours upon activation and/or structural alteration (reviewed in 6,7). In certain tumours activation of proto-oncogenes resulted from chromosomal translocations, leading to overexpression of e.g. the *c-myc* gene in Burkitt lymphoma (8,9) or leading to a chimeric protein with altered function, like the *bcr-abl* fusion gene product in chronic myelogenous leukemia (10,11). Additionally, activation may also result from gene amplification (e.g. *c-myc* in particular tumours) or from point mutations in e.g. the H-*ras* gene in bladder carcinomas. Structural alteration, which in the case of the cellular homologue of the *v-erbB* gene leads to a truncated epidermal growth factor (EGF) receptor protein, is yet another manner of proto-oncogene activation (12).

Although several oncogenes have been identified by their potential to transform cells in culture, experiments indicate that contribution of a single oncogene to tumorigenesis is limited. Rather the concerted action of two or more oncogenes seems to be needed for full transformation of non-immortalized cells in culture (13). The need for multiple oncogenes is further supported by the findings in crosses from transgenic mice carrying a single oncogene, where the presence of two mutations increases the incidence of cancer (reviewed in 14). From the studies with cells in culture it is held that a primary oncogenic event may lead to a block in differentiation, allowing the cells to proliferate continuously. Aneuploidy that may result from such continued replication may subsequently facilitate acquisition of a second oncogenic mutation in these immortalized cells, that further contributes to carcinogenesis. The *in vivo* relevance of the idea that multiple factors are needed for full transformation, is supported by statistical findings. Based on the notion that the frequency of cancer increases with age, it has been suggested that many distinct steps are needed for (at least part of the) tumours to develop to full malignancy (15). The distinct clinical manifestations of colon carcinoma offered the possibility to study the sequence of changes that occur during progression of this type of cancer (16). Although these oncogenic events often occur in a certain order, it seems that in general accumulation rather than a specific order is important. This multi-step character of tumorigenesis is also illustrated by the molecular events leading to tumour development in transgenic models of fibrosarcomas of the skin and of the β-cells in the islets of Langerhans (17).

Relationship between oncogenes and growth-stimulating molecules

The list of proto-oncogenes that are involved in tumorigenesis after activation has become extensive in the last years. Nearly every cellular gene that stimulates growth, may be termed an oncogene when it becomes constitutively expressed. A striking feature about cellular (proto)-oncogenes is, that the products encoded by many of them were found to be identical to already known polypeptide growth factors, growth factor receptors or components of growth-stimulating signal transduction pathways. This supported the suggestion that tumour cells can stimulate their own growth in an autocrine way. An extended version of the autocrine growth stimulation model has been

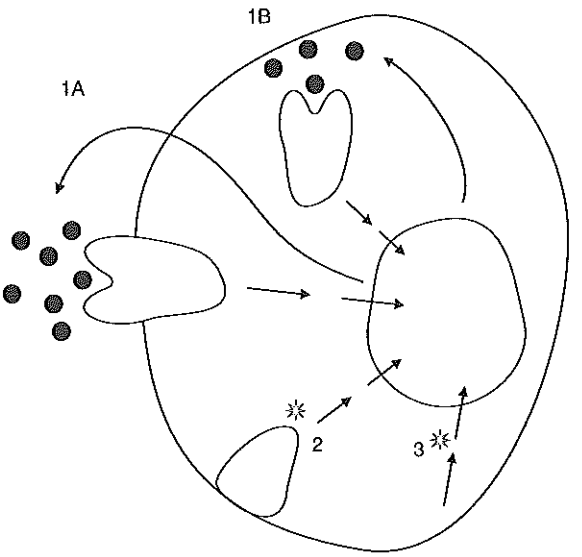

Figure 1. A schematic model of the contribution of factors to uncontrolled growth in tumour cells. Mitogenic signals in the nucleus may result from the extracellular (1a) or intracellular (1b) interaction of a growth factor and its appropriate receptor (autocrine growth stimulation); from expression of a structurally altered growth factor receptor, which signals independently of exogenous stimuli (2); or from the aberrant expression of intracellular signal tranducing components (3). Adapted from Heldin (69).

proposed to explain the ways in which oncogenes may contribute to unrestrained proliferation. In this model growth may result from several different aberrations. Firstly, from deregulated expression of growth factors by the tumour cells, which then should express the appropriate receptors as well. Secondly, from expression of structurally altered growth factor receptors, that for example act independently of exogenous stimuli. And finally, from aberrant expression of intracellular components, that function in transducing growth-stimulating signals from the membrane to the nucleus (Figure 1).

Cell-derived oncogenes can be classified according to the functions of their gene products, which share homology with known cell products (Table 1). Oncogenes that encode growth factors, growth factor receptor tyrosine kinases, and non-receptor tyrosine kinases form one category. In this group the *c-sis* oncogene has become the paradigmatical growth factor-encoding oncogene, as in several studies the platelet-derived growth factor (PDGF) B-chain gene was found to be the human homologue of the transforming *v-sis* gene of simian sarcoma virus (SSV). This was based on the high similarity between these genes and their gene products (18-20). The Parodi-Irgens feline sarcoma virus was also found to contain a gene that shares homology with PDGF B-chain (21). The observation of sequence similarity was an indication for the involvement of PDGF in SSV transformation. As inhibition by PDGF antibodies or by suramin, that prohibits growth factor/receptor interactions, reverted SSV transformation (22,23), it was suggested that PDGF plays a role in this transformation, possibly in an autocrine manner. Likewise, in case of constitutive expression in certain tumour cells, the cellular *sis* oncogene probably also mediates its transforming capacity by stimulating growth in an autocrine way. A more detailed description of PDGF, its biological functions and its role in tumorigenesis is given in Chapter 1.2.

Components acting in signal transduction pathways constitute another category with the *ras* and *raf* oncogenes as the best-known members. Activation of the p21Ras

TABLE 1. Categories of cellular oncogenes with examples of representative members.

growth factors, receptor and non-receptor tyrosine kinases

oncogene	function	references
sis	B-chain of PDGF	18,19
erbB	truncated EGF receptor	12
fms	mutant CSF-1 receptor	57
src	non-receptor tyrosine kinase	58,59

signal transduction pathway components

oncogene	function	references
ras	membrane-associated GTPase	60
raf	protein serine/threonine kinase	61,62
mos	protein serine/threonine kinase	63,64

nuclear factors

oncogene	function	references
jun	transcription factor; part of AP-1	25,65
fos	transcription factor; part of AP-1	26,66
myc	sequence-specific DNA binding	67,68
rel	transcription factor	27

Abbreviations used are: EGF: epidermal growth factor; CSF-1: colony-stimulating factor-1; GTP guanosine triphosphate.

GTPase has been shown to cause translocation of the Raf gene product to the membrane. Raf, in turn, phosphorylates serine and threonine residues in another signal transduction enzyme MAPK kinase, which eventually leads to phosphorylation of transcription factors in the nucleus (24).

The nuclear oncogenes form a special group of oncogenes as their gene products act as transcriptional regulators of several target genes. The gene products of the *c-jun* and *c-fos* oncogenes have thus been identified as components of the transcription factor AP-1 (25,26), whereas the protein encoded by *c-rel* has been found homologous to the transcription factor NF-κB (27). Deregulated or constitutive expression of a transcription factor-encoding oncogene can thus extend the effect of a single oncogenic event by inducing transcription of several genes containing DNA-binding sequences for that factor. Due to protein-protein interactions that sometimes occur between different transcription factors, the effects evoked by oncogenic events in these factors may be even more extensive.

Tumour suppressor genes

Tumour suppressor genes contribute to carcinogenesis after losing their growth-restraining functions upon mutation (reviewed in 28-30). Less than a dozen tumour suppressor genes have been described thus far, but many more will probably be uncovered in the coming years. In contrast to the earlier described oncogenes, where the presence of only one mutated allele is sufficient, mutational events must occur in both alleles for a tumour suppressor gene to contribute to abnormal growth. Deletions in tumour suppressor genes occur frequently in several tumour types. In general a small deletion or point mutation in a tumour suppressor gene is often the first hit followed by loss of heterozygosity (LOH) of the gene or chromosome region containing the other allele, such that only the mutant allele remains. Homozygous deletions are often involved as well.

Evidence for the contribution of genetically altered tumour suppressor genes to tumorigenesis has come from three different observations. Somatic cell hybrids between

tumorigenic and non-tumorigenic cells, did not cause induction of tumour formation after injection, unless the hybrid cells lacked specific chromosomes derived from the normal cell (reviewed in 31). This suggested the presence of genes on these chromosomes that suppressed tumour-promoting activity of their genetically altered counterparts. Secondly, the study of familial cancers suggested that neoplastic transformation may involve alterations in growth restraining genes (tumour suppressor genes) that are inherited as constitutional recessive mutations (32). LOH finally, has been shown to facilitate identification of chromosome regions that may uncover recessive tumour suppressor gene mutations through chromosome loss, recombination or gene conversion.

Mutations in one allele of a tumour suppressor gene can be inherited. They play a role as dominant predispositions to cancer. This is different from the oncogenes that play only a minor role in predisposition to cancer, as their inheritance through the germline could be lethal, due to their dominant nature. A proportion of several tumours actually occurs in familial forms. One of the most obvious examples is retinoblastoma, in which the hereditary form may account for 40% of cases. By contrast, in the other 60% of cases, no history of retinoblastoma could be found in the family. In 1971 Knudson formulated his two-hit mutational hypothesis to explain this phenomenon. In the familial form individuals were thought to have inherited a germline mutation in the retinoblastoma (RB) susceptibility gene, which was followed by a somatic mutation in the second allele of this tumour suppressor gene, thus leading to tumour formation (32). In the sporadic form two somatic mutations in the two alleles of the RB gene were found to give rise to retinoblastoma tumour formation.

Identified tumour suppressor genes

To date, only a limited number of tumour suppressor genes that are involved in certain tumour types, has been identified in detail (Table 2). The list is still growing, as other putative tumour suppressor genes have been postulated based on frequent deletions of chromosome regions in certain tumour types. However, the sequences and functions of these candidate tumour suppressor genes are often still unknown. Mutations in the RB and p53 genes have been detected in many types of tumours, thus suggesting their involvement in a wide range of malignancies. The fact that the proteins encoded by these genes are ubiquitously expressed is indeed consistent with their involvement in several different tumour types.

In case of p53, missense mutations, especially in the region between amino acid 100 and 300, are often observed (33,34), followed by LOH of the other wild-type gene copy. These missense mutations result in production of mutant p53 proteins, that exhibit an altered conformation, that interferes with the ability of p53 to bind specific DNA sequences (35,36) and to activate transcription (37,38). Some groups have shown that overexpression of wild-type p53 caused cell cycle arrest in the G_1 phase (39,40), whereas others demonstrated restriction of cell proliferation and induction of programmed cell death (apoptosis) as well (41,42). Lane (43) proposed a model for p53 action that takes into account these findings. According to this model, genes that are regulated by p53 inhibit DNA replication in order to repair DNA after it has been damaged, whereas apoptosis is induced if DNA repair appears unsuccessful. This

TABLE 2. Identified tumour suppressor genes and their possible involvement in tumorigenesis.

gene	locus	(putative) function	tumour type	references
RB	13q14	sequestration of transcription factor	several types (a.o. retinoblastoma, osteosarcoma, breast, bladder, cervix prostate carcinoma, leukemia)	70-75
p53	17p13	transcription factor	several types (a.o. breast, lung, colon, bladder, liver carcinoma)	76-78
WT1	11p13	transcription factor	Wilms tumour malignant mesothelioma	79-81 82,83
NF1	17q11	GAP-like activity	neurofibrosarcoma	84,85
APC	5q21	cell-adhesion	colorectal carcinoma	86,87
DCC	18q21	cell-adhesion	colorectal carcinoma	88
p16	9p21	CDK4 inhibitor	several types (a.o. melanoma, glioma, breast, bladder carcinoma)	46,47

defence mechanism would be disturbed by loss-of-function mutations in the p53 gene and this would eventually lead to cancer. The identification of CIP1/WAF1 as a p53 target gene fits in with this model as the product encoded by this target gene was found to bind the complex of cyclins and cyclin-dependent kinases (CDKs), thereby prohibiting the cell cycle to continue (44,45). The product of the recently identified p16 gene was shown to be involved in inhibition of one particular CDK, CDK4, which functions in the G_1 phase of the cell cycle. As it was found to be homozygously deleted in many different tumour types, p16 was thus considered a tumour suppressor gene (46,47).

The protein encoded by the RB tumour suppressor gene is differentially phosphorylated during the cell cycle (48). The suppressive capacity that RB demonstrates during G_0 and G_1, when it is underphosphorylated, can be overcome by several mechanisms. The binding of transforming proteins of DNA tumour viruses to hypophosphorylated RB (49-51) and the presence of mutations in this binding domain of the unphosphorylated RB protein (52) both affect RB in its growth-restraining function, possibly by facilitating release of the RB-bound E2F protein (53-55). Upon release from the RB protein, the transcription factor E2F is able to bind and activate cellular proliferation genes. In this way loss-of-function of RB could contribute to carcinogenesis.

Several other tumour suppressor genes, like the NF1, APC, DCC and WT1 genes, have been reported to be involved in only a single or a few tumour types (see Table 2). Mutations in NF1 were observed in von Recklinghausen's neurofibromatosis, characterized by abnormal proliferation of neural crest cells and, in more severe forms, by neurofibrosarcomas. The APC and DCC genes that are frequently lost in colon

carcinomas, both encode proteins that are likely to be involved in cell-adhesion. Finally, the WT1 tumour suppressor gene is one of the (at least three) genes that contributes to development of Wilms tumours, one of the most common pediatric solid malignancies. WT1 encodes a transcription factor that binds to gene promoters containing the core binding sequence 5'-GCG GGG GCG-3' (56). In Chapter 5.1 the WT1 gene and gene product are presented in more detail, as this binding sequence is also present in the promoter region of the PDGF A-chain gene.

1.2 PLATELET-DERIVED GROWTH FACTOR (PDGF)

Platelet-derived growth factor genes and gene products

Platelet-derived growth factor (PDGF) was discovered as a major constituent of blood serum with high mitogenic activity for mesenchymal cell types like smooth muscle cells and fibroblasts. It was first isolated from human platelets (89,90), but soon observed to be produced by many other cell types as well (see later). Initial characterization of PDGF from platelets revealed that it is a 30 kDa dimeric molecule composed of two highly homologous but distinct polypeptide chains, denoted A- and B-chain (91). Although PDGF-AB proved to be the major dimeric isoform of platelets (70%), homodimeric PDGF-BB was also found (92,93). The PDGF-AA homodimer has been identified in several human tumour cell lines (94-96). The A- and B-chains are encoded by different genes which have been mapped to chromosomes 7p22 (97-99) and 22q13.1 (100,101), respectively. The high structural similarity in exon/intron boundaries between the PDGF A- and B-chain genes suggests that they stem from a common ancestral gene.

Three PDGF A-chain transcripts of 1.9, 2.3 and 2.8 kb have been identified which result from alternative promoter and polyadenylation signal use (97). Furthermore, alternative splicing at exon 6 in the 3' end of these transcripts gives rise to short and long splice variants that exhibit different C-termini (102,103). The long variant thereby obtains a stretch of basic amino acids in its C-terminal end. The PDGF B-chain gene predominantly encodes a 3.5 kb transcript, but in some cell types smaller transcripts of about 2.6 kb have been detected. These seem to be activated from a distinct promoter, possibly at the end of the first exon (104) or in the first intron (RPH Dirks, pers. comm.). If this is indeed the case, the 5'-untranslated sequence (UTS) that causes translation inhibition would be missing in the smaller transcript, which would result in better translation (105).

In Chapter 1.4 the factors and regulatory regions involved in PDGF A- and B-chain mRNA expression as well as in expression of the later to be introduced PDGF receptor genes are reviewed.

PDGF dimeric structure, processing and secretion

The dimeric structure of PDGF is important for its biological function, as activity is lost after chemical reduction of the molecule. Consistent with their homology in gene structure, the mature PDGF A- and B-chain polypeptides share about 60% amino acid homology, the positions of the eight cysteine residues being perfectly conserved. These

cysteine residues are involved in intrachain and interchain disulphide bridges that eventually determine the dimeric structure of PDGF. Intrachain bonds are formed between the 1st and 6th, 3rd and 7th and 5th and 8th residues whereas the 2nd and 4th cysteine residues form the interchain bond in the dimers (106-109), thereby contributing to the stability of the protein (110). Analysis of the crystal structure of homodimeric PDGF-BB confirmed these intra- and interchain bonds between the various cysteine residues (111). The importance of this structure was further underscored by the high topological similarity of other dimeric growth factors like nerve growth factor (NGF) and transforming growth factor-β2 (TGF-ß2) (112).

Biosynthesis and processing of PDGF was studied in transfection studies using cDNA constructs (113,114). The A- and B-chain precursor forms both enter the secretory pathway because of their hydrophobic leader sequences (20,97). The PDGF-AA isoform is subsequently dimerized as a 40 kDa factor that becomes efficiently secreted as a 30 kDa protein after N-terminal processing. The PDGF-AB heterodimer is also secreted, though less efficiently (115), as a molecule of about 30 kDa. The 56 kDa PDGF-BB homodimer undergoes N- and C-terminal processing giving rise to a minor secreted product of 30 kDa. However, due to further processing a major form of 24 kDa can also be observed, that is not secreted but remains cell-associated (in the Golgi-system or in the endoplasmic reticulum) due to a retention signal in the C-terminal part of the PDGF B-chain propeptide (116,117). This retention motif is homologous to the basic stretch of amino acids encoded by exon 6 of the earlier mentioned PDGF A-chain long splice variant, raising the interesting possibility that the long PDGF A-chain variant is also intracellularly accumulated. V-*sis* and PDGF A-chain constructs in which the signal sequences mediating secretion were mutated, were localized in the nucleus due to this exon 6-encoded amino acid sequence, which then acts as a nuclear targeting signal (118,119). Furthermore, the retention motif has also been shown to target PDGF A-chain (long) and B-chain molecules to the cell matrix, at least partly through binding to glycosaminoglycans (120-123). The matrix could thus provide a scaffold for PDGF A-chain (long) and B-chain dimeric proteins.

Although the precise biological function of these compartmentalized PDGF molecules is not fully clear yet, it seems that differential compartmentalization constitutes an important way to regulate PDGF action.

PDGF receptors

In order to exert its function, PDGF has to bind to specific high-affinity receptors. Upon receptor binding, a large number of cell-specific effects, like chemotaxis, synthesis of extracellular matrix components and mitogenesis are evoked. The use of purified or recombinant forms of all three PDGF dimers has revealed that there has to be more than one PDGF receptor type (124,125). One of these receptors, designated the α-receptor, was found to bind PDGF-AA, -AB and -BB with high affinity, whereas the ß-receptor was shown to bind PDGF-BB with high affinity and -AB with a tenfold lower affinity. No binding of PDGF-AA could be demonstrated to the latter receptor (125). So the existence of distinct receptor subunits that show different specificities for the various isoforms, provides another possible level of control PDGF activity.

The α- and ß-receptors have been mapped to chromosome 4q (126) and 5q (127),

respectively. The PDGF ß-receptor was cloned first and was found to give rise to a glycosylated protein of 170-185 kDa (128-131). Cloning of the α-receptor was performed soon afterwards (126,132). The PDGF α-receptor was also observed to be post-translationally glycosylated resulting in a molecular weight of about 170 kDa. Both receptors were found to be similar in structure containing an extracellular part with five immunoglobulin-like domains which mediate growth factor binding, a transmembrane domain, and an intracellular domain with protein tyrosine kinase activity. This tyrosine kinase domain is split by a so-called kinase insert domain which has no kinase activity. High amino acid homology between the two receptor subtypes especially exists in the juxtamembrane and tyrosine kinase regions.

In experiments using a kinase-negative receptor mutant, the intrinsic kinase activity of the PDGF ß-receptor was shown to be essential for PDGF-induced processes in the cell (133). Despite the kinase-inhibiting mutation, this mutant could, however, still be internalized and degraded upon ligand binding. A ligand-induced dimerization mechanism comparable to that for the EGF receptor was proposed, to account for activation of the intracellular kinase of the PDGF receptor upon ligand binding (134). Indeed, incubation of PDGF-BB with purified PDGF ß-receptors was found to result in dimerization of these receptors, as revealed by cross-linking agents (135). Dimerization subsequently leads to activation of the kinase, probably through an *in trans* autophosphorylation of the two receptor subunits (136). This is further strengthened by the observation that ß-receptors lacking the intracellular domain inhibits activation of wild-type PDGF ß-receptors (137).

The notion of the importance of dimerization for PDGF receptor activity has led to the PDGF receptor subunit model (138; see also Table 3). In this model dimeric PDGF αα-receptors are predicted to be formed by binding of PDGF-AA, -AB and -BB, αß-receptors by PDGF-AB and -BB and ßß-receptors by PDGF-BB. The ability of PDGF-AB to bind PDGF ß-receptors in cells only expressing this receptor subtype has been a point of discussion in several studies. Downregulation of PDGF α-receptors by PDGF AA-treatment in fibroblasts (139) or the absence of detectable amounts of PDGF α-receptor in fibroblasts and smooth muscle cells (140,141) did not seem to affect PDGF-AB binding, suggesting an interaction between PDGF-AB and the PDGF ßß-receptor dimer. However, Van Zoelen et al. (142) clearly demonstrated that pretreatment with PDGF-AA not only resulted in decreased binding of PDGF-AB, but also in decreased PDGF-AB-induced proliferation of NRK cells. Furthermore, others showed that in ß-receptor transfected cells (143,144) and in cells showing a deleted α-receptor (145) PDGF-AB bound with much lower affinity than BB, possibly only through a monovalent interaction between the B-chain and the ß-receptor.

TABLE 3. Binding of dimeric PDGF isoforms by dimeric PDGF receptors.

	PDGF isoform		
PDGF receptor	AA	AB	BB
αα	+	+	+
αß	-	+	+
ßß	-	-	+

By epitope mapping and site directed mutagenesis it has become clear that certain residues within the region 25-37 of the mature PDGF-BB molecule (= 106-118 of the PDGF B-chain propeptide and 136-148 of the

v-sis sequence) are probably involved in PDGF ß-receptor binding (146-149). Interestingly, these identified residues are localized in a single loop of amino acids at the external face of the crystallized dimer (111). Other studies using PDGF A/B chimeras largely confirmed the importance of these residues but stressed the importance of the externally located residues 54-63 for B-chain activity as well (150,151). A conserved basic region in the PDGF A-chain (residues 159-161) was demonstrated to be involved in PDGF α-receptor binding (152). Studies with mutant PDGF α-receptors showed that the second and third Ig-like domains are involved in high affinity binding of PDGF-AA, and that the first three domains are needed for binding of BB (153-155). The ligand binding region of the ß-receptor was mapped to the first three Ig-like domains as well (156,154).

Intracellular or extracellular PDGF receptor activation?

The transforming potential of PDGF B-chain/c-*sis* is comparable to that of v-*sis* (157,158), whereas the A-chain transforms less efficiently (113,159). Furthermore, the v-*sis* and PDGF B-chain translation products are assembled and processed in a similar way. There is still discussion, whether the PDGF- or v-*sis*-induced PDGF receptor activation and subsequent transformation take place extracellularly or in an intracellular compartment. The fact that antibodies directed to PDGF did not always revert a transformed phenotype (22) favours the possibility of intracellular activation. Furthermore, the addition of an endoplasmic reticulum retention signal to the PDGF B-chain sequence did not abrogate its transforming capacity (160). Other data conflict with the idea of intracellular autocrine interaction, as intracellular activation failed to lead to increased c-*fos* expression (161) and suramin was shown to reverse transformation without affecting intracellular receptor activation (23,386). Moreover, the endoplasmic signal turned out to be more of a retrieval signal than a retention signal (162). Retention in the trans-Golgi still abrogated transformation, suggesting the need for translocation to the membrane (162).

According to a model that was proposed to account for all these findings (163), intracellular formation of a ligand/receptor complex may be sufficient for receptor autophosphorylation. However, in this model a mitogenic response only occurs after translocation of this complex to the cytoplasmic membrane for proper substrate association. It might still be that responses which are not involved in growth and transformation, could result from activation of the PDGF receptor without actual translocation. Compartmentalization of PDGF autocrine signal transduction pathways has indeed been suggested in c-*sis*-transformed NIH 3T3 cells, with several signal transduction molecules being activated in the presence of suramin and others not (164). An intriguing observation is further that a pool of ligand-bound PDGF ß-receptors remained tyrosine phosphorylated after internalization, suggesting an active role for the intracellular ligand/receptor complex in substrate phosphorylation (165).

Signal transduction and PDGF-inducible genes

Dimerization and subsequent autophosphorylation of PDGF receptors leads to the binding of various intracellular substrates to these phosphorylated tyrosine residues which serve as attachment sites. Binding is mediated through conserved motifs in the

TABLE 4. Substrate binding to autophosphorylated tyrosine residues in the human PDGF ß-receptor.

Y(P)[a]	substrate	references
579	Shc	173
579	Src-family	247
581	Src-family	247
716	Grb-2	175
740	PI3-K	248
751	PI3-K	248,249
751	Nck	250
740	Shc	173
751	Shc	173
771	Shc	173
771	GAP	248,251
778	unknown	
857	unknown	
1009	PLCγ	170,252 253
1021	PLCγ	170,252-254
1009	PTP-1D/Syp SH-PTP2/PTP-2C	255,256

Abbreviations used (as far as mentioned in the literature) are: GAP: GTPase activating protein; Grb: growth factor receptor bound; PI3-K: phosphoinositol 3-kinase; PLCγ: phospholipase Cγ; PTP: phosphotyrosine phosphatase.

a. Y(P): (auto)phosphorylated tyrosine residues in the human PDGF ß-receptor

substrates, the so-called Src homology 2 (SH2) domains. Y751 and Y857 were identified as the two major sites of ligand-induced phosphorylation of the human PDGF ß-receptor (166). Mutation of these residues resulted in impaired mitogenic stimulation (167). In these mutant receptors impaired substrate binding may be due to a generally lowered (aspecific) binding, rather than to decreased specific substrate binding at Y857. Substrates interacting with identified phosphorylated tyrosine residues in the human PDGF ß-receptor are listed in Table 4.

Analysis of signal transduction pathways in cells transfected with PDGF α- or ß-receptors has revealed that in both cases cells respond mitogenically to stimulation with the proper PDGF isoform (168). Mitogenic responses are probably mediated in parallel through several substrates that bind PDGF ß-receptor tyrosine residues. PI3-K (phosphatidylinositol-3-kinase) and PLCγ (phospholipase Cγ) have been implicated to play a role in this process (169), though others failed to show a necessary role for PLCγ in Y1009- or Y1021-mutated PDGF ß-receptors (170). However, residual PLCγ binding to the non-mutated tyrosine could not be excluded in the latter report. Furthermore, Src-like kinases were suggested to be required for proper PDGF-induced mitogenicity as well (171). Substrates like Shc and Grb-2, that have been found associated with PDGF ß-receptors, act as adaptor molecules mediating binding and activation of other molecules. The binding sites for Grb-2 have recently been identified. Grb-2 activates the Sos/Ras signaling pathway by binding to phosphotyrosine-bound Shc or PTP-1D/Syp (172-174) or via direct binding to phosphotyrosine residues in the PDGF ß-receptor (175). Activation of p70 S6 kinase seems to contribute to the PDGF-induced mitogenic signal as well (176). As this pathway is independent of Ras, other mechanisms are probably involved in mitogenic signaling as well.

Studies with cells expressing chimeric PDGF α-receptor or FGF-1 receptor molecules containing the ß-receptor kinase insert revealed that migration towards PDGF and circular actin reorganization require the binding of PI3-K to ß-receptor kinase insert tyrosine residues, and possibly the activation of the small GTP-binding protein Rac (177-179). The observed association of PI3-K and focal adhesion kinase (FAK) after PDGF stimulation raises the intriguing possibility of cross-talk between these pathways leading to changes in cell morphology (180). Kundra et al. (181) found migration-promoting (PLCγ and PI3-K) as well as migration-suppressing (GAP, GTPase-activating protein) substrates in PDGF ß-receptor-mediated chemotaxis.

PDGF α- and ß-receptors activate both common and unique signaling pathways

(168). Association with PLCγ and PI3-K occurred with similar stoichiometry, but the binding affinity for GAP was much lower in case of the PDGF α-receptor (182). Formation of the second messenger phosphatidic acid was shown to be increased by stimulation of ß-receptor compared to α-receptor-induced pathways (183). Furthermore, in heterodimeric form the α-receptor was found to be phosphorylated on Y754, whereas no phosphorylation of this residue was seen in αα- or ßß-homodimers, suggesting binding of a specific protein (184).

PDGF-induced intracellular signal transduction eventually leads to the expression of several genes, which further mediate the responses initiated by PDGF. The best known are the so-called immediate early genes. They form a large group of genes whose expression is induced several minutes to hours after stimulation by growth factors, like PDGF (reviewed in 185,186). The *c-fos*, *c-jun*, *c-myc* and EGR1 genes are examples of these early genes. Being (part of) transcription factors, their gene products have been implicated to play a role in proliferative responses by regulating processes associated with DNA synthesis and cell division. PDGF has been shown to control the activity of Fos and Jun gene products at the transcription level (reviewed in 187,188), but also by regulating Fos protein stability (189). An intriguing observation was that PDGF was also capable of increasing cyclin D1 expression, thereby providing a direct link between external growth factor-mediated stimulation and regulation of transition through the G_1 phase of the cell cycle (190,191).

Ligand-mediated endocytosis and subsequent degradation is a mechanism to downregulate the growth factor receptor-mediated biological response. Phosphorylation of Y579 was shown to be essential for endocytosis of the PDGF ß-receptor (192), whereas others showed that residues Y740 and Y751 were indispensable (193). Analysis of purified stimulated PDGF receptors revealed covalent binding of ubiquitin (127). Ubiquitin is thought to play a role in mediating intracellular protein degradation. Indeed ligand-induced polyubiquitination was observed in PDGF ß-receptors, depending upon kinase activity of the receptor and most probably phosphorylation of residues Y1009 and Y1021 (194). Mitogenic signalling was repressed through accelerated intracellular degradation of ß-receptors after polyubiquitination.

PDGF in normal cell types and in non-malignant disorders

PDGF has been implicated to be involved in human placental development (195,196), whereas a role in mouse embryonic development was suggested as well (197-199). Evidence for the latter came from a study in which PDGF B-chain knock-out mice were found to die around birth, suffering from renal, hematological, and cardiovascular abnormalities (200). A similar phenomenon was seen in PDGF ß-receptor-deficient mice, although it was suggested that the role of the ß-receptor may be partly masked because of compensation by the α-receptor (201). Expression of the PDGF α-receptor was seen in several mesodermal tissues during mice and rat embryonic stages as well as in some ectoderm-derived tissues later in development (202,203). The availability of Patch (Ph) mutant mice carrying a deletion in the PDGF α-receptor may be helpful in further clarifying the role of the PDGF α-receptor subunit during mammalian development (204).

Furthermore, a role for PDGF (especially PDGF-AA/PDGF α-receptor) was proposed

in development of neuronal tissue. The developmental expression pattern of PDGF A-chain and PDGF α-receptor detected in the rat and mouse central nervous system (CNS) suggests that PDGF has an important function in development of the oligodendrocytic lineage (198,205,206). Neurotrophic activity of PDGF-BB acting via the PDGF ß-receptor was shown to result in neuronite outgrowth (207). Similarly, detection of PDGF and PDGF receptors in the peripheral nervous system (PNS) of neonatal and adult rats indicated a possible role for PDGF in development and maintenance of the PNS as well (208).

After the first description in platelets, production of PDGF was observed in a whole range of other cell types as well. Cytotrophoblasts in human placenta (195), endothelial cells (209-212), arterial smooth muscle cells (213,214), keratinocytes (215), alveolar epithelial cells (216), and monocytes and activated macrophages (217-219) were all found to be capable of synthesizing one or both PDGF chains. Not in all these cell types the function of the produced PDGF is fully understood. In the case of cytotrophoblasts and smooth muscle cells an autocrine function was suggested as both cell types also respond to PDGF (220).

PDGF is a potent growth factor and chemoattractant for connective tissue cells, like fibroblasts, and is also chemotactic for inflammatory cells (221,222). The role PDGF plays in wound healing processes was explained by the capacity to attract connective tissue cells and subsequently stimulate them to produce extracellular matrix components. The expression of PDGF in the healing wound and of PDGF ß-receptors on capillary endothelial and vascular smooth muscle cells (223-225) together with a (weak) angiogenic activity of PDGF (226), further underscored this involvement in wound healing. An actual wound healing effect of recombinant PDGF-BB was demonstrated in chronic pressure ulcer patients (227). This process was mediated through PDGF-BB-induced increases in fibrobast proliferation and differentiation (228).

PDGF produced by alveolar macrophages was implicated to be involved in normal lung architecture, possibly by stimulating fibroblasts to produce extracellular matrix (ECM) components (216,229). Excessive production of PDGF by these intra-alveolar macrophages may lead to idiopathic pulmonary fibrosis, a lung disorder characterized by large fibrotic areas (216). Another example of a non-malignant proliferative disorder in which PDGF is involved, is atherosclerosis. PDGF-induced proliferation of smooth muscle cells is thought to contribute to the thickening seen in the intima of affected vessels in this disease (230,231). Likewise, PDGF was mentioned to play a role in the inflammatory process in rheumatoid synovitis, possibly by stimulation of proliferation in the vasculature (232).

PDGF in oncogenesis

Temporally and spatially deregulated expression of PDGF and its receptor may not only give rise to non-malignant proliferative disorders, like idiopathic pulmonary fibrosis and atherosclerosis, but may contribute to tumorigenesis in certain malignancies as well.

The putative involvement of deregulated PDGF chain and/or receptor expression in the transformation of certain cell types has been highlighted in several reports (Table 5), although in most cases the actual contribution of upregulation of either of these genes

TABLE 5. PDGF chain and receptor (mRNA and/or protein) expression in various human tumour cell lines.

cell type	expression of PDGF chain (A/B) and/or receptor (α/ß)[a]	references
glioma	PDGF A, B, α, ß	257,258
	PDGF A, B, α, ß	233
large cell lung carcinoma	PDGF A, B	259
	PDGF ß	260
malignant mesothelioma	PDGF A, B	261
	PDGF A, B, ß	262,263
mammary carcinoma	PDGF A, B	264
melanoma	PDGF A, B	95
	PDGF A, (B)	265
prostate carcinoma	PDGF A, B	266
sarcoma (several types)	PDGF A, B, α, ß	267
	PDGF A, B, α, ß	233
thyroid carcinoma	PDGF α, ß	268

a. In case several cell lines of a certain type were analyzed, not every cell line expressed all indicated chains and receptors.

has not been proven. Co-expression of PDGF A- and/or B-chain and its appropriate PDGF receptor, as observed in e.g. glioma, malignant mesothelioma, and sarcoma cell lines, is highly suggestive of an autocrine loop of growth stimulation. Actual support for the existence of an autocrine growth stimulation loop came from the demonstration of PDGF receptor activation in the absence of exogenous ligand in e.g. sarcoma cell lines (233). Furthermore, although PDGF receptors initially were thought to be expressed by mesenchymal cells only, clear expression was seen in some cell lines of epithelial origin (lung carcinoma, thyroid carcinoma) as well. If this is the same *in vivo*, this receptor expression might thus confer a growth advantage to the tumour cells compared to their normal counterparts that do not possess PDGF receptors. Finally, in those tumour cell lines that only express one or both PDGF chains and no PDGF receptors, PDGF may contribute to tumorigenesis by exerting a paracrine effect on stromal cells *in vivo*. Stomal cells are known to play a sustaining role in tumorigenesis.

As studies using tumour-derived cell lines are not always representative for the situation *in vivo*, the expression of PDGF and/or its receptors was also studied in primary tumour samples (Table 6). In several tumours putative autocrine growth stimulation loops (PDGF A/α-receptor in gliomas, ovarian carcinomas and PDGF B/ß-receptor in choriocarcinomas, fibrosarcomas, lung carcinomas) were postulated, based on the co-expression of ligand and receptor. Several studies, particularly those on gliomas (234,235), have suggested that tumour-derived PDGF may be involved in a paracrine way in stromal development, as stromal cells in the primary tumour were found to express the PDGF ß-receptor. Further support for this comes from recent experiments in which mice were xenografted with PDGF B-chain-transfected melanoma cells (236). In tumours derived from these transfected melanoma cells, neovascularization and stromal development were clearly observed, whereas the absence of PDGF B-chain resulted in necrotic areas, thus suggesting an important role for B-chain-induced stromal development in tumorigenesis.

TABLE 6. PDGF chain and receptor (mRNA and/or protein) expression in primary tumour material and surrounding stroma.

tumour type	expression of PDGF chain (A/B) and/or receptor (α/ß)[a]	references
basal cell carcinoma	PDGF A, B, α, ß	269
choriocarcinoma	PDGF (A), B, ß	270
colorectal carcinoma	PDGF[a] and PDGF ß	271
fibrosarcoma	PDGF (A), B, (α), ß	272
glioma	PDGF A, B, α, ß	234
	PDGF α, ß	273
	PDGF ß	235
lung carcinoma	PDGF B, ß	274
ovarian carcinoma	PDGF[a] and PDGF α, ß	275
	PDGF[a]	276
sarcoma (several types)	PDGF B	277

a. No discrimination between PDGF-AA and -BB could be made, as the antibodies could not discriminate between the various PDGF isoforms.

Interference in the PDGF-mediated response; antagonists

Studies were initiated to find antagonists to interfere in the PDGF-mediated responses. Several compounds were found to inhibit PDGF function, either in specific or in non-specific ways. Suramin, that non-specifically prevents ligand/receptor interactions, was shown to inhibit *v-sis*-induced transformation (23). Neomycin was found to act as a PDGF isoform- and receptor-specific antagonist in transfected porcine endothelial cells by inhibition of PDGF-BB/PDGF α-receptor interactions; binding and activation of the α-receptor by PDGF-AA or the ß-receptor by PDGF-BB were not affected (237). Small compounds called tyrphostins have recently been described as representatives of a novell class of selective tyrosine kinase blockers, especially for the PDGF and stem cell factor receptors (238). A peptide corresponding to part of the PDGF B-chain protein inhibited the ability to bind and subsequently activate both PDGF α- and ß-receptors (239). Given the importance of a dimeric structure for PDGF functioning, monomeric PDGF would be predicted to act as a PDGF antagonist as well. However, analysis of monomeric forms of PDGF, created by mutation of the cysteine residues forming the interchain bridges, revealed that these monomers acted as agonists rather than antagonists (107); dimerization of receptors apparently still occurs in this situation.

In several cell types signal transduction through PDGF receptors was impaired after transfection of truncated α- or ß-receptors (137,240,241). In conditioned medium of a human osteosarcoma cell line a soluble form of the PDGF α-receptor was found which seemed to result from proteolytic clipping of the intact protein (242). The function of this soluble receptor is unclear, but it may regulate the PDGF response, either by regulating growth factor levels or by protecting growth factors from degradation.

Antibodies directed against PDGF (22,243) or the PDGF α-receptor (244) were found to prevent binding of PDGF to their receptors, thereby blocking the autocrine loop. In view of the observations that PDGF probably activates its receptors intracellularly as well, antibodies may however not be useful to block this process in all situations. The construction of a non-receptor binding PDGF A-chain mutant, PDGF-0,

which still forms heterodimers, was thus of great importance. Indeed, introduction of this PDGF-0 in *c-sis*/PDGF-B transformed fibroblasts resulted in a reversal of the transformed phenotype by the formation of PDGF-0B heterodimers (245). In a similar approach Shamah et al. (246) developed dominant-negative PDGF ligand mutants that were found to revert the transformed phenotype of human astrocytoma cells. Future studies will have to reveal whether antibodies or antagonists are of any clinical use in preventing or inhibiting PDGF-mediated diseases, including malignancies and non-malignant disorders.

1.3 TRANSCRIPTION AND TRANSCRIPTION FACTORS

The role of transcription in gene expression

Transcription constitutes the first stage of gene expression resulting in the production of primary transcripts from particular genes. As the initial event in the process of regulation of gene expression, transcription thus plays an important role in determining the temporal and tissue-specific production of gene products. This first step is subsequently followed by several post-transcriptional processes, like splicing, translation, and protein modification. The whole process of transcription can be divided into three distinct processes: initiation, elongation and termination. At initiation, enzymes bind to a particular gene to start RNA synthesis, whereas in turn at termination the enzymes dissociate from the DNA. In the elongation phase the enzymes move along the DNA, thereby extending the RNA transcript.

Machinery for basal transcription

Initiation of transcription involves the interaction of a multimeric protein complex with the promoter region of the gene. A critical step in basal transcription is the assembly of these multimers, that consist of RNA polymerase and several general transcription initiation factors. In eukaryotes three distinct types of nuclear RNA polymerases, that are involved in transcription of particular gene sets, were identified. Class I RNA polymerases are involved in transcription of the genes encoding the large rRNA precursor, class III in snRNA and tRNA genes, whereas RNA polymerase II is reponsible for synthesis of mRNA precursors.

Based on several studies a model for assembly of the RNA polymerase II transcription initiation complex has emerged (278-280). According to this model (Figure 2), an initial binding complex is formed between transcription factor IID (TFIID) and the TATA box in the gene promoter. TFIID itself is a complex consisting of the TATA-binding protein (TBP) and several TBP-associated factors (TAFs). It appears that for basal transcription, an interaction between TBP and the TATA box is sufficient to recruit other necessary factors. In contrast, the entire TFIID complex (including the TAFs) is needed for interaction with upstream acting factors in activated transcription. Therefore, a role for at least some of the TAFs as co-activators was proposed (281). The TATA box-TFIID or TATA box-TBP complexes act as scaffolds for TFIIB-binding, which subsequently leads to recruitment of RNA polymerase II and TFIIF. Together these factors form the minimal initiation complex, that acts in separating the DNA strands at

the transcription initiation site. Factor TFIIA is not part of the minimal complex that is essential for basal transcription, but appears to inhibit binding of transcriptional repressors to TFIID. Binding of TFIIE and TFIIH to the minimal complex results in the formation of the complete initiation complex.

Studies on basal transcription have mainly concerned TATA-containing promoters. However, there are also genes, e.g. those encoding TGF-ß (282) and the EGF receptor (283), that do not contain TATA sequences, but instead use initiator elements around the transcription start site. These initiators may be recognized by one of the TAFs or by specific initiator-binding proteins (reviewed in 284). Furthermore, the fact that at least some of these TATA-less promoters (e.g. of the WT1 gene) are relatively GC-rich suggests the involvement of factors binding GC boxes as well (285).

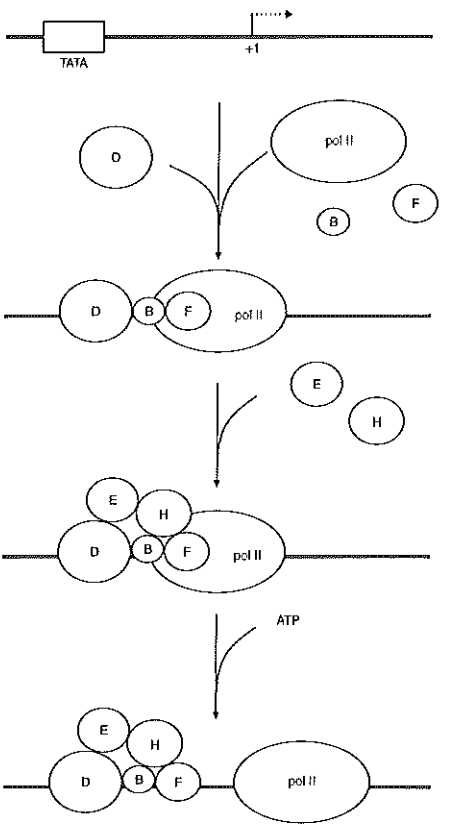

Figure 2. Schematic model of transcription initiation complex assembly. The various transcription factors needed for transcription initiation complex formation are indicated by their characters. Pol II: RNA polymerase II. Position +1 indicates the transcription start site and TATA represents the typical TATA box sequence, located around position -30 to -20. See also text for detailed description. Adapted from Buratowski (280).

Activation of basal transcription: transcription factors

The basal transcription as mediated by RNA polymerase II and several transcription initiation factors can be regulated by specific transcription factors that bind *cis*-acting regulatory elements in the promoter (also called upstream promoter elements) and enhancer regions (bidirectional elements assisting in initiation of transcription) of a gene. Transcription factors influencing the process of basal transcription can be divided into two categories: those that act as transcriptional activators and those that act as transcriptional repressors. The latter can also act as repressors of activated transcription by interfering with the ability of transcriptional activators to stimulate transcription (see later).

In order to exert their function, transcription factors must bind gene regulatory regions in a sequence-specific way. However, DNA binding alone is not sufficient for regulation. Rather the interaction with other factors and/or with the basal transcription machinery (TBP, TAFs, TFIIB) is needed for modulation of transcription. Transcription

TABLE 7. Functional domains of transcription factors.

domain	function	examples of factors	references
homeodomain	DNA binding	Oct-1	303
basic	DNA binding	Fos, Jun	304,305
		E47	306
Cys-His zinc-finger	DNA binding	Sp1	307
		WT1	56
Cys-Cys zinc-finger	DNA binding	v-ErbA	308
high mobility group	DNA binding	HMG-1	309
		LEF-1	310
leucine zipper	di/oligomerization	Fos, Jun	311,312
helix-loop-helix	di/oligomerization	E12, E47	306,313
acidic	transactivation	glucocorticoid receptor	314
Pro-rich	transactivation	WT1	315
Glu-rich	transactivation	Sp1	316
		WT1	315

factors are composed of several distinct domains that mediate the DNA binding, oligo- or dimerization, and transactivation functions (Table 7). It is the combination of domains that contributes to the specificity of transcription factors in regulating expression of particular genes. Protein-protein interactions between transcriptional activators and other (basal) transcription factors like TFIID and the TAFs play an essential role in activation of transcription. However, that leaves the problem as to how transcription of these factors is regulated. It appears that in many cases transcription factors are ubiquitously expressed, but that these are inactive forms of the factors. Conversion of these inactive forms into active forms of the transcription factors results in transcription of particular genes in certain cell types or in response to specific stimuli. Several distinct mechanisms may be involved in this process. Ligand binding results in activation of e.g. the glucocorticoid receptor acting as a transcription factor (reviewed in 286). Disruption of an associated inhibitory protein as in the case of IκB bound to transcription factor NF-κB (287) provides another means of activation. A third way to activate transcription factors is protein modification by e.g. phosphorylation (reviewed in 288,289).

In at least some cases the activating potential of enhancers appears to require the formation of so-called stereospecific nucleoprotein complexes, which confer further specificity to the complex process of gene regulation in eukaryotic cells (reviewed in 290). In these three-dimensional complexes the interactions between several sequence-specific transcription factors play an essential role. In e.g. the IFN-ß enhancer complex the transcription factor HMG I(Y) functions as an architectural (bridging) component mediating protein-protein interactions between other transcription factors that bind to this enhancer (291).

Transcriptional repressors

Although positive regulation was emphasized in eukaryotic cells, it has now become clear that transcriptional repressors constitute an integral part of the process of transcription regulation as well. Repressors can act at nearly every step in transcription initiation, i.e. by interfering with nuclear localization, with activator/DNA binding, with the general transcription machinery and with formation of the pre-initiation complex

(reviewed in 292,293).

Repressors that function in downregulating the activity of one or more positively acting transcription factors can roughly be divided into two classes: passive and active repressors. Passive repressors act in distinct ways to downregulate transcriptional activation. Competition for DNA binding sites or steric hindrance because of overlapping DNA binding sites, as seen in repression of oestrogen-induced transcription of the lactoferrin gene by COUP-TF (294), may be a way to prevent binding and activity of positively regulating transcription factors. Protein-protein interactions between activators and repressors may lead to sequestration of activators, resulting in reduced activation of transcription. This inhibition, known as squelching (295), acts independent of DNA binding and is achieved through the transactivation domains (e.g. the transrepression of oestrogen-induced transcription by c-*fos* or c-*jun*). Active repressors downregulate transcription directly rather than by inhibition of transactivators. Although this process is not fully understood yet, it seems that active repressors interfere with formation of the transcription initiation complex or promote binding of inhibiting factors to basal transcription factors like TFIID.

Other mechanisms regulating gene expression

There are other mechanisms that can influence the transcription process in addition. One of them is the organized chromatin structure in eukaryotes (reviewed in 296). DNA is associated with octameric structures of several distinct histone proteins (H2A, H2B, H3, H4). Together these so-called nucleosomes form the basic units of the chromatin. Positioning of nucleosomes can have a repressive effect on transcription by occlusion of binding sites for transcription initiators or transcription factors. This phenomenon is known as transcriptional silencing. Transcription activators may act to disrupt binding of these nucleosomes, thereby relieving this repressive effect. Modification of the histone proteins influences the interaction between DNA and nucleosomes as well. Histone acetylation has been suggested to facilitate transcription by destabilization of nucleosomes (297), whereas transient deacetylation of in particular H2A and H2B histones may improve binding of regulatory proteins in the initial activation of transcription (298). The nucleosomal structure can also facilitate the transcription process by bringing widely dispersed regulatory sequences in the DNA in juxtaposition (reviewed in 299). DNA topology in the form of local supercoiled domains plays a role in eukaryotic transcription as well (300). The nuclear matrix is considered a dynamic structural part of the nucleus that is involved in processes like replication, recombination and also gene expression. In the latter case the nuclear matrix may place structural constraints on the promoter regions of certain genes thereby facilitating transcription factor accessibility (301).

Methylation of CpG sites in protein-binding regulatory elements can be a way to repress or inhibit transcription. Promoters of transcriptionally active genes are generally hypomethylated, whereas transcriptionally inactive genes often contain hypermethylated promoter regions. A special form of transcriptional repression mediated by methylation is seen in a phenomenon called genomic imprinting (reviewed in 302). Imprinting is the exclusive expression of a particular gene from either the maternal (e.g. the H19, IGF2R genes) or the paternal (e.g. the IGF2 gene) chromosome, the other gene copy being

transcriptionally repressed. In this process expression of one of the gene copies is accompanied by differential methylation of these gene copies.

1.4 REGULATION OF PDGF CHAIN AND RECEPTOR mRNA EXPRESSION

Regulation of the biological action of PDGF

As already mentioned in Chapter 1.2, PDGF is involved in many physiological (e.g. neuronal development, wound healing) and pathological (e.g. idiopathic pulmonary fibrosis, atherosclerosis, neoplasia) processes. The biological action of PDGF and the responsiveness to PDGF are determined by regulation of the expression levels of PDGF chains and PDGF receptors in the cell types involved. In this whole process, activation of PDGF chain and receptor gene transcription constitutes the first, and for this reason important, level of regulation. In addition, there are several other levels of regulation that further determine PDGF availability. Examples include alternative PDGF B-chain promoter use leading to transcripts lacking 5' translation inhibition sequences, alternative splicing of PDGF A-chain and PDGF α-receptor transcripts, degradation of PDGF chain and receptor messengers, translation, dimerization, and compartmentalization.

Factors regulating PDGF chain and receptor mRNA expression

Because of the important role activation of transcription plays in determining availability of PDGF ligand and receptor, many factors have been studied for their involvement in this process (Table 8). However, it should be stressed that in most studies the effects on the steady-state mRNA levels, representing effects on both transcription rate and messenger stability, were analyzed. Some of these factors may act directly in activation of transcription, whereas in other situations there may be an (post-transcriptional) effect on messenger half-life. Collectively, the data obtained in these studies indicate that regulation occurs in a cell type-specific way, as for example TPA modulates PDGF B-chain gene expression in endothelial cells and monocytes, but does not seem to do so in smooth muscle cells. Furthermore, different external stimuli may demonstrate opposite effects in a certain cell type. An example is the TNF-α-induced upregulation and IFN-γ-induced downregulation of the PDGF chain genes in endothelial cells.

Other factors and mechanisms have been implicated in regulation of PDGF chain and receptor gene expression in specific cell types as well. Angiotensin II-induced hypertrophy of vascular smooth muscle cells was associated with an increase in the PDGF A-chain steady-state mRNA level (317). Oxidized forms of low density lipoproteins, known to be important in atherosclerosis, were found to act via induction of PDGF A-chain gene expression in smooth muscle cells (318). Hypoxia-dependent vasoconstriction mediated by endothelial cells correlates with observations that PDGF is a strong vasoconstrictor and that oxygen is a potent regulator of PDGF B-chain transcription in endothelial cells (319). PDGF B-chain gene expression in endothelial cells was suggested to be regulated by fluid shear stress via a *cis*-acting element in its promoter as well (320,321). Furthermore, PDGF receptor mRNA expression in smooth

TABLE 8. Factors regulating PDGF chain and/or receptor mRNA levels in various cell types.

factor	cell type[a]	effect on mRNA expression level of PDGF chains and receptors[b]	references
PDGF-AA	fibroblasts	α-receptor -; ß-receptor -	365
	melanoma cells	A-chain ↑	366
PDGF-BB	fibroblasts	α-receptor ↑; ß-receptor ↑	365
	mesangial cells	A-chain ↑; B-chain ↑	367
TGF-ß	endothelial cells	A-chain ↑; B-chain ↑	368,369
	fibroblasts	A-chain ↑; B-chain -	327,370
	fibroblasts	α-receptor ↓; ß-receptor -	370
	(mouse) fibroblasts	B-chain ↑	324
	(rat) mesangial cells	B-chain ↑; ß-receptor ↑	326
	myofibroblasts (Ito cells)	A-chain ↑	329
	osteoblastic cells	α-receptor ↓	371
	smooth muscle cells	A-chain ↑; α-receptor ↓	331
	smooth muscle cells	A-chain ↑; B-chain -	372
	(rat) smooth muscle cells	A-chain ↑; B-chain -	328
	(rabbit) smooth muscle cells	B-chain ↑; ß-receptor ↑	325
phorbol ester (TPA)	erythroleukemia cells	B-chain ↑	373,374
	endothelial cells	A-chain ↑; B-chain ↑	368
	monocytes	A-chain ↑; B-chain ↑	375
	smooth muscle cells	A-chain ↑; B-chain -	372
aFGF	endothelial cells	A-chain ↑; B-chain -	376
	smooth muscle cells	A-chain ↑; B-chain -	372
bFGF	endothelial cells	B-chain ↓	377
	(bovine) smooth muscle cells	α-receptor ↑; ß-receptor -	378
cAMP	endothelial cells	A-chain ↓; B-chain ↓	368,369
	glioma/osteosarcoma cells	B-chain ↓	379
IL-1	smooth muscle cells	A-chain ↑	380
	fibroblasts	A-chain ↑	380
	endothelial cells	A-chain ↑; B-chain ↑	381
IL-2	peritoneal macrophages	A-chain ↑; B-chain ↑	382
TNF-α	endothelial cells	A-chain ↑; B-chain ↑	381,383
	smooth muscle cells	A-chain ↑; B-chain ↑	372
IFN-γ	endothelial cells	A-chain ↓; B-chain ↓	381
	alveolar macrophages	B-chain ↑	384
dexamethasone	(rat) hepatoma cells	A-chain ↓	385
	alveolar macrophages	B-chain ↑	384

Abbreviations used are: a/b FGF: acidic/basic fibroblast growth factor; cAMP: cyclic adenosine monophosphate; IFN-γ: interferon-γ; IL: interleukin; TGF-ß: transforming growth factor-β; TNF-α: tumour necrosis factor-α; TPA: 12-O-tetradecanoyl phorbol 13-acetate.
a. Unless clearly indicated, cells were of human origin.
b. ↑ upregulation; ↓ downregulation; - no effect.

muscle cells and fibroblasts has been shown to be influenced by culturing (322). The PDGF receptor subunit expression in cultured mesangial cells was found to be influenced by the spatial organization of the ECM, although it is not clear whether the mRNA levels were also affected (323).

Regulation by TGF-ß (transforming growth factor-ß)

TGF-ß is probably one of the most extensively studied regulators of PDGF chain and receptor expression. This may have to do with the observation that stimulation of proliferation by TGF-ß, as generally seen in mesenchymal cells, is in many cases accompanied by an effect on transcription of the PDGF chain genes. It has been shown

that TGF-ß-stimulated proliferation of fibroblasts, vascular smooth muscle cells or (rat) mesangial cells was accompanied by upregulation of PDGF ß-receptor and/or PDGF B-chain mRNA and protein expression (324-326). Others suggested that TGF-ß-stimulated proliferation was mediated by an increase in PDGF A-chain mRNA and protein in cell types like foreskin fibroblasts, vascular smooth muscle cells, and myofibroblasts (327-329).

In general, addition of TGF-ß results in inhibition of growth of epithelial cells. The density-dependent inhibiting effect of TGF-ß in cultured human fibroblasts, involving downregulation of PDGF α-receptors, is another example (330). A model has been presented for TGF-ß-induced bimodal proliferation in smooth muscle cells. At low concentrations TGF-ß was found to stimulate growth, possibly by induction of PDGF A-chain expression, whereas at higher concentrations the PDGF effect seemed to be decreased by downregulation of PDGF α-receptor expression (331).

However, TGF-ß-induced growth stimulation seemed only partly dependent on induction of PDGF expression. In a few reports both PDGF-AA-dependent and independent mechanisms were shown to mediate this stimulation (332,333). Two other observations further support a PDGF-independent effect. Firstly, the growth-inhibiting effect of TGF-ß in mammary epithelial cells was accompanied by induction of PDGF B-chain transcription as well (334). Secondly, the phenotypic transformation of NRK cells by TGF-β was not paralleled by enhanced production of PDGF (335).

Molecular mechanisms involved in PDGF A-chain transcription

Several studies have been performed to identify regulatory regions that are involved in basal and induced activation of PDGF A- and B-chain transcription (for a recent review see 336). These (partly cell type-specific) PDGF A-chain and B-chain regulatory elements are schematically depicted in Figure 3.

All three identified PDGF A-chain transcripts were found to arise from a single transcription start site, roughly 26 bp downstream of a TATA sequence in the PDGF A-chain promoter region (102,337). The region between bp -618 and +392, especially the highly GC-rich -150/-33 region which contained more than 80% of this activity, was found to be sufficient for full promoter activity in HeLa cells (338). In several studies this GC-rich region was found to be S1 nuclease sensitive, suggesting the existence of a local single-stranded DNA region. Deletion of the GC-motif decreased S1 nuclease sensitivity and also PDGF A-chain promoter activity (339,340). Sp1 and Egr-1 binding sites that contain many C and G nucleotides could be identified in this region. Furthermore, in mesangial cells a phorbol ester-induced protein binds to a DNase I protected sequence (-102/-82) in this region as well (341). Indeed, basal transcription seems to be enhanced through binding of nuclear proteins to Sp1-like consensus sequences in the -73/-46 region of the PDGF A-chain promoter, as determined in renal epithelial cells (342). Binding of transcription factor WT1 to sequences that are identical to Egr-1 binding sequences, was shown to affect PDGF A-chain activity in HeLa cells as well (343,344). Furthermore, other S1 nuclease sensitive regions that demonstrated regulating activity in transfection studies were observed downstream (+50/+67) and further upstream (-513/-482) of the transcription start site in both HeLa and A172 glioblastoma cells (345,346). A serum response element (SRE; -477/-468) was also

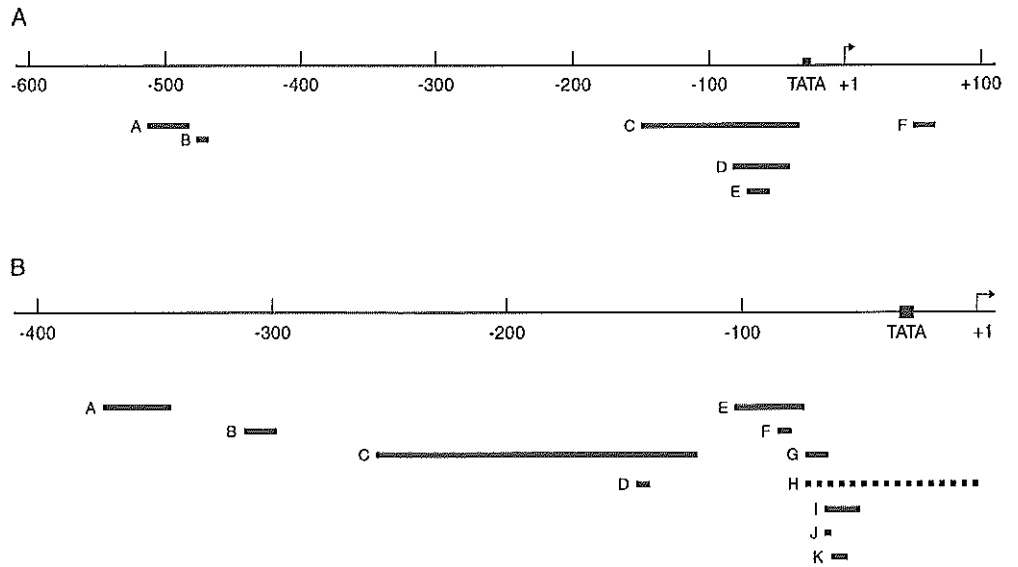

Figure 3. A. Identified regulatory regions in the PDGF A-chain promoter and 5' untranslated sequence (UTS). Boxes represent regulatory sequences at the indicated positions from the transcription start site. A (-513/-482; 346), B (-477/-468; 338), C (-150/-33; 338), D (-82/-70; 340), E (-72/-55; 339), F (+50/+67; 345). TATA (-31/-27). **B.** Identified regulatory regions in the PDGF B-chain promoter and 5' UTS. Boxes represent regulatory sequences at the indicated positions from the transcription start site. A (-372/-343; 353), B (-312/-298; 359), C (-255/-119; 359), D (-145/-139; 353), E (-103/-73; 353,354), F (-92/-86; 355), G (-80/-70; 354,355), H (-72 and further; 353), I (-64/-45; 354), J (-64/-61; 358), K (-61/-54; 355,357,358). TATA (-30/-24).

identified (338).

The GC-rich PDGF A-chain promoter contains many potential CpG methylation sites. Transcriptional activity of the PDGF A-chain gene appeared to be regulated by methylation, which suggests that methylation plays an important role in PDGF A-chain expression (347). Repression of transcription was implicated in regulation of PDGF A-chain messenger expression as well. A cell type-specific suppressive element was described in the first intron of the PDGF A-chain gene, which functioned in HeLa, but not in glioblastoma cells (348,349). An additional negative regulatory element was identified in the -1800/-812 region (342). Furthermore, a region in the 5' untranslated sequence (UTS) (+99/+184) was suggested to act as a post-transcriptional repressor of A-chain mRNA expression in a rhabdomyosarcoma cell line (350).

Molecular mechanisms involved in PDGF B-chain transcription

The 5' UTS of the PDGF B-chain messenger seems to be involved in inhibition of translation (351,105). However the fact that a retrovirus expressing high levels of the entire PDGF B-chain messenger is tumorigenic in mice (352), suggests that the translation inhibition can be overcome by strong transcription. This underscores the importance of activation of transcription for PDGF B-chain biological action.

Sequence analysis of the 5' flanking region of the PDGF B-chain transcription start site revealed the presence of several potential binding sites for transcription factors. Although binding to either of two Sp1 binding sites has not been demonstrated directly, one of them (-145/-139) lies within a region that is involved in positive regulation of TPA-induced PDGF B-chain expression in K562 cells (353). However, others showed that mutation of this site did not influence the TPA-induced expression (354). ETS-like (-80/-70) and AP-1-like (-92/-86) binding sites were also identified in reporter assays in endothelial cells (355). Although being part of a positive regulatory region (-103/-73), deletion of this AP-1-like sequence did not affect activation of transcription in TPA-treated K562 cells (353). The negative regulatory region (-372/-343) that these authors observed in K562, could not be confirmed by others in these or other cells. Dirks et al. (356) showed that there was no effect on transcription regulation effect when the promoter was confined to 112 bp upstream of the transcription start. A minimal promoter region of the PDGF B-chain gene in TPA-induced and uninduced in K562 cells extended 42 bp upstream of the TATA box (i.e. to bp -72) (353). By linker scanning analysis a small region (-64/-45) was identified as being especially important in this respect in TPA-treated K562 cells (354). By combining reporter gene analysis with gel mobility shift assays and *in vivo* footprinting analysis the CCACCCAC element (-61/-54) was identified as a binding site for a strong transcriptional activator, possibly Sp1, in osteosarcoma, endothelial, and TPA-treated K562 cells (355,357,358). Furthermore, in their study Dirks et al. (358) identified another PDGF B-chain-promoter element, a TCTC sequence (-64/-61), as (part of) a weak transcriptional activator in untreated K562 cells and prostate carcinoma (PC3) cells. In choriocarcinoma (JEG-3) cells this same region was shown to mediate transcriptional activity as well, but due to chromosomal DNA methylation, the promoter does not seem to be accessible for transactivators in this cell type *in vivo* (358).

In other cell types, like glioblastoma (A172) and osteosarcoma (HOS), other regions (-312/-298 and to a lesser extent -255/-119) were shown to be involved in positive regulation of basal PDGF B-chain expression (359). In the same cells HTLV-I and II transactivator proteins induced PDGF B-chain expression via enhancer sequences located between bp -1393 and +74.

Regions in the first intron and downstream and far upstream of the transcription unit have been implicated in activation of PDGF B-chain gene transcription as well. DNaseI hypersensitive (DH) sites in the first intron were shown to silence basal PDGF B-chain expression in osteosarcoma (U2-OS) cells, but to increase expression in choriocarcinoma (JEG-3) cells (360). In contrast, Dirks et al. (356) found that the same first intron sequences decreased B-chain expression in these JEG-3 cells. Moreover, downregulation of PDGF B-chain promoter activity by this sequence was also observed in cervix (HeLa) and prostate (PC3) carcinoma cells. In the same paper a cell type-specific activator was identified roughly 25 kb of the transcription start. This activator may work in PC3 and TPA-treated K562 cells *in vivo*, as it co-localized with a DH site in these cells (356). Furthermore, DH sites at -8.6 kb and -9.9 kb showed strong enhancer activity in TPA-treated K562 cells when tested apart and in combination. In HeLa and PC3 cells the individual sites showed low enhancer activity, but when combined in a larger fragment this activity was lost almost completely (361).

Activation of PDGF B-chain transcription is a very complex, cell type-specific process involving both positive and negative regulatory elements located upstream, downstream and inside the transcription unit.

Molecular mechanisms involved in PDGF receptor transcription

Only a limited amount of data is available from studies on regulation of PDGF α-receptor transcription, whereas up till now nothing has been published on activation of PDGF ß-receptor gene transcription. Concerning the PDGF α-receptor recently two reports have been published describing the molecular cloning of the murine (362) and human (363) α-receptor promoter. The 93 bp minimal promoter region of the murine PDGF α-receptor was found to function in a way that mirrored the tissue-specific expression of this gene. In the human gene a similar but slightly larger promoter region was identified (363). Alternative promoter use (within intron 12) in teratocarcinoma cells was shown to result in novel PDGF α-receptor transcripts (364). Molecular cloning of the human PDGF ß-receptor promoter and upstream regulatory sequences will probably follow soon. Future studies will possibly reveal cell type-specific enhancing and repressive elements of both PDGF α- and ß-receptor genes.

Collectively the beforementioned data thus suggest that regulation of PDGF A- and B-chain expression and probably also of PDGF α- and β-receptor expression should be studied in the cell type of interest as in many studies cell type-specific regulating elements have been identified.

1.5 REFERENCES

1. Kaplan PL, Anderson M, Ozanne B. Transforming growth factors production enables cells to grow in the absence of serum: an autocrine system. Proc. Natl. Acad. Sci. USA 79: 485-489, 1982.
2. Temin HM. In: Growth regulating substances for animal cells in cultures (Defendi V and Stoker M, eds) vol. 7: 103-116, The Wistar Symposium Monograph, Wistar Institute Press, Philadelphia, 1967.
3. Sporn MB, Todaro GJ. Autocrine secretion and malignant transformation of cells. N. Engl. J. Med. 303: 878-880, 1980.
4. Stehelin D, Varmus HE, Bishop JM, Vogt PK. DNA related to the transforming genes of avian sarcoma viruses is present in normal avian DNA. Nature 260: 170-173, 1976.
5. Reddy EP, Reynolds RK, Santos E, Barbacid M. A point mutation is responsible for the acquisition of transforming properties by the R4 bladder carcinoma oncogene. Nature 298: 147-152, 1982.
6. Bishop JM. The molecular genetics of cancer. Science 235: 305-311, 1987.
7. Weinberg RA. The genetic origins of human cancer. Cancer 61: 1963-1968, 1988.
8. Klein G. Specific chromosomal translocations and the genesis of B-cell-derived tumors in mice and men. Cell 32: 311-315, 1983.
9. Leder P, Battey J, Lenoir G, Moulding C, Murphy W, Potter H, Stewart T, Taub R. Translocations among antibody genes in human cancer. Science 222: 765-771, 1983.
10. Heisterkamp N, Stephenson JR, Groffen J, Hansen PF, De Klein A, Bartram CR, Grosveld G. Localization of the *c-abl* oncogene adjacent to a translocation break point in chronic myelocytic leukemia. Nature 306: 239-242, 1983.
11. Groffen J, Stephenson JR, Heisterkamp N, De Klein A, Bartram CR, Grosveld G. Philadalphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. Cell 36: 93-99, 1984.
12. Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, Ullrich A, Schlessinger J, Waterfield MD. Close similarity of epidermal growth factor receptor and *v-erbB* oncogene protein sequences. Nature 307: 521-527, 1984.

13. Land H, Parada LF, Weinberg RA. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 306: 596-602, 1983.
14. Compere SJ, Baldacci P, Jaenisch R. Oncogenes in transgenic mice. Biochim. Biophys. Acta 948: 129-149, 1988.
15. Peto R, Roe FJC, Lee PN, Levy L, Clack J. Cancer and aging in mice and men. Br. J. Cancer 32: 411-426, 1975.
16. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 61: 759-767, 1990.
17. Christofori G, Hanahan D. Molecular dissection of multi-stage tumorigenesis in transgenic mice. Semin. Cancer Biol. 5: 3-12, 1994.
18. Doolittle RF, Hunkapiller MW, Hood LE, Devare SG, Robbins KC, Aaronson SA, Antoniades HN. Simian sarcoma virus onc gene, *v-sis*, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science 221: 275-277, 1983.
19. Waterfield MD, Scrace GT, Whittle N, Stroobant P, Johnsson A, Wasteson Å, Westermark B, Heldin C-H, Huang JS, Deuel TF. Platelet-derived growth factor is structurally related to the putative transforming protein $p28^{sis}$ of simian sarcoma virus. Nature 304: 35-39, 1983.
20. Josephs SF, Guo C, Ratner L, Wong-Staal F. Human proto-oncogene nucleotide sequences corresponding to the transforming region of Simian sarcoma virus. Science 223: 487-491, 1984.
21. Besmer P, Snyder HW Jr, Murphy JE, Hardy WD Jr, Parodi A. The Parodi-Irgens feline sarcoma virus and simian sarcoma virus have homologous oncogenes, but in different contexts of the viral genomes. J. Virol. 46: 606-613, 1983.
22. Johnsson A, Betsholtz C, Heldin C-H, Westermark B. Antibodies against platelet-derived growth factor inhibit acute transformation by simian sarcoma virus. Nature 317: 438-440, 1985.
23. Betsholtz C, Johnsson A, Heldin C-H, Westermark B. Efficient reversion of simian sarcoma virus-transformation and inhibition of growth factor-induced mitogenesis by suramin. Proc. Natl. Acad. Sci. USA 83: 6440-6444, 1986.
24. Schaap D, Van der Wal J, Howe LR, Marshall CJ, Van Blitterswijk WJ. A dominant-negative mutant of *raf* blocks mitogen-activated protein kinase activation by growth factors and oncogenic p21*ras*. J. Biol. Chem. 268: 20232-20236, 1993.
25. Bohmann D. Transcription factor phosphorylation: a link between signal transduction and the regulation of gene expression. Cancer Cells 2: 337-344, 1990.
26. Rauscher FJ III, Sambucetti LC, Curran T, Distel RJ, Spiegelmann BM. Common DNA-binding site for Fos protein complexes and transcription factor AP-1. Cell 52: 471-480, 1988.
27. Kieran M, Blank V, Logeat F, Vandekerckhove J, Lottspeich F, LeBail O, Urban MB, Kourilsky P, Bäuerle PA, Israel A. The DNA binding subunit of NF-kB is identical to factor KBF1 and homologous to the *rel* oncogene product. Cell 62: 1007-1018, 1990.
28. Marshall CJ. Tumor suppressor genes. Cell 64, 313-326, 1991.
29. Evans HJ, Prosser J. Tumor-suppressor genes: cardinal factors in inherited predisposition to human cancers. Environ. Health Perspect. 98: 25-37, 1992.
30. Levine AJ. The tumor suppressor genes. Annu. Rev. Biochem. 62: 623-651, 1993.
31. Harris H. The analysis of malignancy by cell fusion: the position in 1988. Cancer Res. 48: 3302-3306, 1988.
32. Knudson AG. Mutation and cancer: statistical study of retinoblastoma. Proc. Natl. Acad. Sci. USA 68: 820-823, 1971.
33. Baker SJ, Fearon ER, Nigro JM, Hamilton SR, Preisinger AC, Jessup JM, Van Tuinen P, Ledbetter DH, Barker DF, Nakamura Y, White R, Vogelstein B. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science 244: 217-221, 1989.
34. Nigro JM, Baker SJ, Preisinger AC, Jessup JM, Hostetter R, Cleary K, Bigner SH, Davidson N, Baylin S, Devilee P, Glover T, Collins FS, Weston A, Modali R, Harris CC, Vogelstein B. Mutations in the p53 gene occur in diverse human tumor types. Nature 342: 705-708, 1989.
35. Kern SE, Kinzler KW, Baker SJ, Nigro JM, Rotter V, Levine AJ, Friedman P, Prives C, Vogelstein B. Mutant p53 proteins bind DNA abnormally in vitro. Oncogene 6: 131-136, 1991.
36. Srinivasan R, Roth JA, Maxwell SA. Sequence-specific interaction of a conformational domain of p53 with DNA. Cancer Res. 53: 5361-5364, 1993.
37. Raycroft L, Wu HY, Lozano G. Transcriptional activation by wild type but not transforming mutants of the p53 anti-oncogene. Science 249: 1049-1051, 1990.
38. Kern SE, Pietenpol JA, Thiagalingam S, Seymour A, Kinzler KW, Vogelstein B. Oncogenic forms of p53 inhibit p53 regulated gene expression. Science 256: 827-830, 1992.
39. Martinez J, Georgoff I, Martinez J, Levine AJ. Cellular localization and cell cycle regulation by a temperature-sensitive p53 protein. Gene Dev. 5: 151-159, 1991.

40. Ullrich SJ, Mercer WE, Appella E. Human wild type p53 adopts a unique conformational and phosphorylation state *in vivo* during growth arrest of glioblastoma cells. Oncogene 7: 1635-1643, 1992.
41. Yonish-Rouach E, Resnitzky D, Lotem J, Sachs L, Kimichi A, Oren M. Wild type p53 induces apoptosis of myeloid leukemic cells that is inhibited by interleukin-6. Nature 352: 345-347, 1991.
42. Shaw P, Bovey R, Tardy S, Sahli R, Sordat B, Costa J. Induction of apoptosis by wildtype p53 in a human tumor-derived cell line. Proc. Natl. Acad. Sci. USA 89: 4495-4499, 1992.
43. Lane DP. p53, guardian of the genome. Nature 358: 15-16, 1992.
44. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75: 805-816, 1993.
45. El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsosn R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. WAF-1, a potential mediator of p53 tumour suppression. Cell 75: 817-825, 1993.
46. Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, Stockert E, Day RS III, Johnson BE, Skolnick MH. A cell-cycle regulator potentially involved in genesis of many tumor types. Science 264: 436-440, 1994.
47. Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature 368: 753-756, 1994.
48. Ludlow JW, Shon J, Pipas JM, Livingston DM, DeCaprio JA. The retinoblastoma susceptibility gene product undergoes cell cycle-dependent dephosphorylation and binding to and release from SV40 large T. Cell 60: 387-396, 1990.
49. Whyte P, Buchkovich JJ, Horowitz JM, Friend SH, Raybuck M, Weinberg RA, Harlow E. Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature 334: 124-129, 1988.
50. Whyte P, Williamson NM, Harlow E. Cellular targets for transformation by the adenovirus E1A proteins. Cell 56: 67-75, 1989.
51. DeCaprio JA, Ludlow JW, Figge J, Shew JY, Huang C-M, Lee W-H, Marsilio E, Paucha E, Livingston DM. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell 54: 275-283, 1988.
52. Kaye FJ, Kratzke RA, Gerster JL, Horowitz JM. A single amino acid substition results in a retinoblastoma protein defective in phosphorylation and oncoprotein binding. Proc. Natl. Acad. Sci. USA 87: 6922-6926, 1990.
53. Bagchi S, Weinmann R, Raychaudhuri P. The retinoblastoma protein copurifies with E2F-1, an E1A-regulated inhibitor of the transcription factor E2F. Cell 65: 1063-1072, 1991.
54. Chellappan SP, Hiebert S, Murdryj M, Horowitz JM, Nevins JR. The E2F transcription factor is a cellular target for the RB protein. Cell 65: 1053-1061, 1991.
55. Chittenden T, Livingston DM, Kaelin WG. The T/E1A-binding domain of the retinoblastoma product can interact selectively with a sequence-specific DNA-binding protein. Cell 65: 1073-1082, 1991.
56. Rauscher FJ III, Morris JF, Tournay OE, Cook DM, Curran T. Binding of the Wilms' tumor locus zinc-finger protein to the EGR-1 consensus sequence. Science 250: 1259-1262, 1990.
57. Sherr CJ, Rettenmeir CW, Sacca R, Roussel MF, Look AT, Stanley ER. The *c-fms* proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell 41: 665-676, 1985.
58. Cartwright CA, Eckhardt W, Simon S, Kaplan PL. Cell transformation by $pp60^{c\text{-}src}$ mutated in the carboxy-terminal regulatory domain. Cell 49: 83-91, 1987.
59. Kmiecik TE, Shalloway D. Activation and suppression of $pp60^{c\text{-}src}$ transforming ability by mutation of its primary sites of tyrosine phosphorylation. Cell 49: 65-73, 1987.
60. Barbacid M. *Ras* genes. Annu. Rev. Biochem. 56: 779-827, 1987.
61. Smith MR, DeGudicibus SJ, Stacey DW. Requirement for *c-ras* proteins during viral oncogene transformation. Nature 320: 540-543, 1986.
62. Morrison DK, Kaplan DR, Rapp U, Roberts TM. Signal transduction from membrane to cytoplasm: growth factors and membrane-bound oncogene products increase Raf-1 phosphorylation and associated protein kinase activity. Proc. Natl. Acad. Sci. USA 85: 8855-8859, 1988.
63. Sagata N, Watanabe N, Vande Woude GF, Ikawa Y. The *c-mos* proto-oncogene product is a cytostatic factor responsible for meiotic arrest in vertebrate eggs. Nature 342: 512-518, 1989.
64. Roy LM, Singh B, Gautier J, Arlinghaus RB, Nordeen SK, Maller JL. The cyclin B2 component of MPF is a substrate for the $c\text{-}mos^{xe}$ proto-oncogene product. Cell 61: 825-831, 1990.
65. Angel P, Allegretto EA, Okino ST, Hattori K, Boyle WJ, Hunter T, Karin M. Oncogene *jun* encodes a sequence-specific transactivator similar to AP-1. Nature 332: 166-171, 1988.
66. Distel RJ, Ro H-S, Rosen BS, Groves DL, Spiegelmann BM. Nucleoprotein complexes that regulate gene expression in adipocyte differentiation: direct participation of *c-fos*. Cell 49: 835-844, 1987.

67. Armelin HA, Armelin MCS, Kelly K, Steward T, Leder P, Cochran BH, Stiles CD. Functional role of *c-myc* in mitogenesis response to platelet-derived growth factor. Nature 310: 655-660, 1984.
68. Blackwell TK, Kretzner L, Blackwood EM, Eisenmann RN, Weintraub H. Sequence-specific DNA binding by the c-Myc protein. Science 250: 1149-1151, 1990.
69. Heldin C-H. Structural and functional studies on platelet-derived growth factor. EMBO J. 11:4251-4259, 1992.
70. Lee EY-HP, To H, Shew JY, Bookstein R, Scully P, Lee W-H. Inactivation of the retinoblastoma susceptibility gene in human breast cancers. Science 241: 218-221, 1988.
71. T'Ang A, Varley JM, Chakraborty S, Murphee AL, Fung T-K. Structural rearrangement of the retinoblastoma gene in human breast carcinoma. Science 242: 263-266, 1988.
72. Yokota J, Akiyama T, Fung Y-K, Benedict WF, Namba Y, Hanaoka M, Wada M, Terasaki T, Shimosato Y, Sugimura T. Altered expression of the retinoblastoma (RB) gene in small-cell carcinoma of the lung. Oncogene 3, 471-475, 1988.
73. Bookstein R, Lee EY, Peccei A, Lee W-H. Human retinoblastoma gene: long-range mapping and analysis of its deletion in a breast cancer cell line. Mol. Cell. Biol. 9: 1628-1634, 1989.
74. Varley JM, Armour J, Swallow JE, Jeffreys AJ, Ponder BA, T'Ang A, Fung YK, Brammar WJ, Walker RA. The retinoblastoma gene is frequently altered leading to loss of expression in primary breast tumours. Oncogene 4: 725-729, 1989.
75. Horowitz JM, Park SH, Bogenmann E, Cheng JC, Yandell DW, Kaye FJ, Minna JD, Fryja TP, Weinberg RA. Frequent inactivation of the retinoblastoma anti-oncogene is restricted to a subset of human tumor cells. Proc. Natl. Acad. Sci. USA 87: 2775-2779, 1990.
76. Lane DP, Benchimol S. p53: oncogene or anti-oncogene? Gene Dev. 4, 1-8, 1990.
77. Hollstein M, Sidransky D, Vogelstein B, Harris, C.C. p53 mutation in human cancers. Science 253: 49-53, 1991.
78. Levine AJ, Momand J, Finlay CA. The p53 tumor suppressor gene. Nature 351: 453-456, 1991.
79. Riccardi VM, Sujanski E, Smith AC, Francke U. Chromosomal imbalance in the aniridia-Wilms' tumor association: 11p interstitial deletion. Pediatrics 61: 604-610, 1978.
80. Van Heyningen V, Boyd PA, Seawright A, Fletcher JM, Fantes JA, Buckton KE, Spowart G, Porteous DJ, Hill RE, Newton MS, Hastie ND. Molecular analysis of chromosome 11 deletions in aniridia-Wilms' tumour syndrome. Proc. Natl. Acad. Sci. USA 82: 8592-8596, 1985.
81. Haber DA, Buckler AJ, Glaser T, Call KM, Pelletier J, Sohn RL, Douglass EC, Housman DE. An internal deletion within an 11p13 zinc finger gene contributes to the development of Wilms' tumor. Cell 61: 1257-1269, 1990.
82. Park S, Schalling M, Bernard A, Maheswaran S, Shipley GC, Roberts D, Fletcher J, Shipman R, Rheinwald J, Demetri G, Griffin J, Minden M, Housman DE, Haber DA. The Wilms tumour gene WT 1 is expressed in murine mesoderm-derived tissues and mutated in a human mesothelioma. Nat. Genet. 4: 415-420, 1993.
83. Langerak AW, Williamson KA, Miyagwa K, Hagemeijer A, Versnel MA, Hastie ND. Expression of the Wilms' tumour gene WT 1 in human malignant mesothelioma cell lines and relationship to PDGF A-chain and IGF-II expression. Genes Chromosomes Canc. 12: 87-96, 1995.
84. Cawthon RM, Weiss R, Xu G, Viskochil D, Culver M, Stevens J, Robertson M, Dunn D, Gesteland R, O'Connell P, White R. A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations. Cell 62: 193-201, 1990.
85. Wallace M, Marchuk D, Andersen L, Letcher R, Odeh H, Saulino A, Fountain J, Brereton A, Nicholson J, Mitchell A, Brownstein B, Collins F. Type 1 neurofibromatosis gene: identification of a large trancript disrupted in three NF1 patients. Science 249: 181-186, 1990.
86. Joslyn G, Carlson M, Thliveris A, Albertsen H, Gelbert L, Samowitz W, Groden J, Stevens J, Spirio L, Robertson M. Identification of deletion mutations and three new genes at the familial polyposis locus. Cell 66: 601-613, 1991.
87. Nishisho I, Nakamura Y, Miyoshi Y, Miki Y, Ando H, Horii A, Koyama K, Utsunomiya J, Baba S, Hedge P. Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. Science 253: 665-669, 1991.
88. Fearon ER, Cho KR, Nigro JM, Kern SE, Simons JW, Ruppert JM, Hamilton SR, Preisinger AC, Thomas G, Kinzler KW, Vogelstein B. Identification of a chromosome 18q gene that is altered in colorectal cancers. Science 247: 49-56, 1990.
89. Antoniades HN, Scherr CD, Stiles CD. Purification of human platelet-derived growth factor. Proc. Natl. Acad. Sci. USA 76: 1809-1813, 1979.
90. Heldin C-H, Westermark B, Wasteson Å. Platelet-derived growth factor; purification and partial characterization. Proc. Natl. Acad. Sci. USA 76: 3722-3726, 1979.
91. Johnsson A, Heldin C-H, Westermark B, Wasteson Å. Platelet-derived growth factor: identification of constituent polypeptide chains. Biochem. Biophys. Res. Commun. 104: 66-74, 1982.

92. Stroobant P, Waterfield MD. Purification and properties of porcine platelet-derived growth factor. EMBO J. 12: 2963-2967, 1984.
93. Hammacher A, Hellman U, Johnsson A, Östman A, Gunnarsson K, Westermark B, Wasteson Å, Heldin C-H. A major part of platelet-derived growth factor purified from human platelets is a heterodimer of one A and one B chain. J. Biol. Chem. 263: 16493-16498, 1988.
94. Heldin C-H, Johnsson A, Wennergren S, Wernstedt C, Betsholtz C, Westermark B. A human osteosarcoma cell line secretes a growth factor structurally related to a homodimer of PDGF A-chains. Nature 319: 511-514, 1986.
95. Westermark B, Johnsson A, Paulsson Y, Betsholtz C, Heldin C-H, Herlyn M, Rodeck U, Koprowsky H. Human melanoma cell lines of primary and metastatic origin express the genes encoding the chains of platelet-derived growth factor (PDGF) and produce a PDGF-like growth factor. Proc. Natl. Acad. Sci. USA 83: 7197-7200, 1986.
96. Hammacher A, Nistér M, Westermark B, Heldin C-H. A human glioma cell line secretes three structurally and functionally different dimeric forms of platelet-derived growth factor. Eur. J. Biochem. 176: 179-186, 1988.
97. Betsholtz C, Johnsson A, Heldin C-H, Westermark B, Lind P, Urdea MS, Eddy R, Shows TB, Philpott K, Mellor AL, Knott TJ, Scott J. cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumour cell lines. Nature 320: 695-699, 1986.
98. Morton CC, Bonthron DT, Collins T. Platelet-derived growth factor A chain is assigned to 7p22-p21. Hum. Gene Mapp. 9, 664, 1987.
99. Stenman G, Rorsman F, Huebner K, Betsholtz C. The human platelet-derived growth factor A chain (PDGF A) gene maps to chromosome 7p22. Cytogenet. Cell Genet. 60: 206-207, 1992.
100. Dalla-Favera R, Gallo RC, Giallongo A, Croce CM. Chromosomal localization of the human homolog (*c-sis*) of the simian sarcoma virus onc gene. Science 218: 686-688, 1982.
101. Swan DC, McBride OW, Robbins KC, Keithley DA, Reddy EP, Aaronson SA. Chromosomal mapping of the simian sarcoma virus onc gene analogue in human cells. Proc. Natl. Acad. Sci. USA 79: 4691-4695, 1982.
102. Bonthron DT, Morton CC, Orkin SH, Collins T. Platelet-derived growth factor A chain: Gene structure, chromosomal location, and basis for alternative mRNA splicing. Proc. Natl. Acad. Sci. USA 85: 1492-1496, 1988.
103. Rorsman F, Bywater M, Knott TJ, Scott J, Betsholtz C. Structural characterization of the human platelet-derived growth factor A-chain cDNA and gene: alternative exon usage predicts two different precursor proteins. Mol. Cell. Biol. 8: 571-577, 1988.
104. Fen Z, Daniel TO. 5' Untranslated sequences determine degradative pathway for alternate PDGF B/c-sis mRNAs. Oncogene 6: 953-959, 1991.
105. Rao CD, Pech M, Robbins KC, Aaronson SA. The 5' untranslated sequence of the c-*sis*/platelet-derived growth factor 2 transcript is a potent translational inhibitor. Mol. Cell. Biol. 8: 284-292, 1988.
106. Jaumann M, Hoppe V, Tatje D, Eichner W, Hoppe J. On the structure of platelet-derived growth factor AA: C-terminal processing, epitopes, and characterization of cysteine residues. Biochemistry 30: 3303-3309, 1991.
107. Andersson M, Östman A, Bäckström G, Hellman U, George-Nascimento C, Westermark B, Heldin C-H. Assignment of interchain disulfide bonds in platelet-derived growth factor (PDGF) and evidence for agonist activity of monomeric PDGF. J. Biol. Chem. 267: 11260-11266, 1992.
108. Hanlu M, Rohde MF, Kenney WC. Disulfide bonds in recombinant human platelet-derived growth factor BB dimer: characterization of intermolecular and intramolecular disulfide linkages. Biochemistry 32: 2431-2437, 1993.
109. Östman A, Andersson M, Bäckström G, Heldin C-H. Assignment of intrachain disulfide bonds in platelet-derived growth factor B-chain. J. Biol. Chem. 268: 13372-13377, 1993.
110. Prestrelski SJ, Arakawa T, Duker K, Kenney WC, Narhi LO. The conformational stability of a non-covalent dimer of a platelet-derived growth factor-B mutant lacking the two cysteines involved in interchain disulfide bonds. Int. J. Peptide Prot. Res. 44: 357-363, 1994.
111. Oefner C, D'Arcy A, Winkler FK, Eggimann B, Hosang M. Crystal structure of human platelet-derived growth factor BB. EMBO J. 11: 3921-3926, 1992.
112. Murray-Rust J, McDonald NQ, Blundell TL, Hosang M, Oefner C, Winkler F, Bradshaw RA. Topological similarities in TGF-ß2, PDGF-BB, and NGF define a superfamily of polypeptide growth factors. Structure 1: 153-159, 1993.
113. Bywater M, Rorsman F, Bongcam-Rudloff E, Mark G, Hammacher A, Heldin C-H, Westermark B, Betsholtz C. Expression of recombinant PDGF A- and B-chain homodimers in Rat-1 cells and human fibroblasts reveal differences in protein processing and autocrine effects. Mol. Cell. Biol. 8: 2753-2762, 1988.

114. Östman A, Thyberg J, Westermark B, Heldin C-H. PDGF-AA and PDGF-BB biosynthesis: proprotein processing in the Golgi complex and lysosomal degradation of PDGF-BB retained intracellularly. J. Cell Biol. 118: 509-519, 1992.
115. May M, Aaronson SA, LaRochelle WJ. Platelet-derived growth factor AB heterodimer interchain interactions influence secretion as well as receptor binding and activation. Biochemistry 32: 11734-11740, 1993.
116. LaRochelle WJ, May-Siroff M, Robbins KC, Aaronson SA. A novel mechanism regulating growth factor association with the cell surface: identification of a PDGF retention domain. Gene Dev. 5: 1191-1199, 1991.
117. Östman A, Andersson M, Betsholtz C, Westermark B, Heldin C-H. Identification of a cell retention signal in the B-chain of PDGF and in the long splice version of the A-chain. Cell Regul. 2: 503-512, 1991.
118. Lee BA, Maher DW, Hannink M, Donoghue DJ. Identification of a signal for nuclear targeting in platelet-derived growth factor-related molecules. Mol. Cell. Biol. 7: 3527-3537, 1987.
119. Maher DW, Lee BA, Donoghue DJ. The altenatively spliced exon of the platelet-derived growth factor A-chain encodes a nuclear targeting signal. Mol. Cell. Biol. 9: 2251-2253, 1989.
120. Khachigian LM, Owensby DA, Chesterman CN. A tyrosine peptide representing the alternatively spliced exon of the platelet-derived growth factor A-chain binds specifically to cultured cells and interferes with binding of several growth factors. J. Biol. Chem. 267: 1160-116, 1992.
121. Raines EW, Ross R. Compartmentalization of PDGF on extracellular binding sites dependent on exon-6 encoded sequences. J. Cell Biol. 116: 533-543, 1992.
122. Kelly JL, Sánchez A, Brown GS, Chesterman CN, Sleigh MJ. Accumulation of PDGF B and cell-binding forms of PDGF A in the extracellular matrix. J. Cell Biol. 121: 1153-1163, 1993.
123. Andersson M, Östman A, Westermark B, Heldin C-H. Characterization of the retention motif in the C-terminal part of the long splice form of platelet-derived growth factor A-chain. J. Biol. Chem. 269: 926-930, 1994.
124. Hart CE, Forstrom JW, Kelly JD, Seifert RA, Smith RA, Ross R, Murray MJ, Bowen-Pope DF. Two classes of PDGF receptor recognize different isoforms of PDGF. Science 240: 1529-1531, 1988.
125. Heldin C-H, Bäckström G, Östman A, Hammacher A, Rönnstrand L, Rubin K, Nistér M, Westermark B. Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types. EMBO J. 7: 1387-1393, 1988.
126. Matsui T, Heidaran M, Miki T, Popescu N, LaRochelle W, Kraus M, Pierce J, Aaronson S. Isolation of a novel receptor cDNA established the existence of two PDGF receptor genes. Science 243: 800-803, 1989.
127. Yarden Y, Escobedo JA, Kuang W-J, Yang-Feng TL, Daniel TO, Tremble PM, Chen EY, Ando ME, Harkins RN, Francke U, Fried VA, Ullrich A, Williams LT. Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. Nature 323: 226-232, 1986.
128. Hart CE, Seifert RA, Ross R, Bowen-Pope DF. Synthesis, phosphorylation, and degradation of multiple forms of the platelet-derived growth factor receptor studied using a monoclonal antibody. J. Biol. Chem. 262: 10780-10785, 1987.
129. Keating MT, Williams LT. Processing of the platelet-derived growth factor receptor. J. Biol. Chem. 262: 7932-7937, 1987.
130. Claesson-Welsh L, Eriksson A, Morén A, Severinsson L, Ek B, Östman A, Betsholtz C, Heldin C-H. cDNA cloning and expression of a human platelet-derived growth factor (PDGF) receptor specific for B-chain-containing PDGF molecules. Mol. Cell. Biol. 8: 3476-3486, 1988.
131. Gronwald RGK, Grant FJ, Haldeman BA, Hart CE, O'Hara PJ, Hagen FS, Ross R, Bowen-Pope DF, Murray MJ. Cloning and expression of a cDNA coding for the human platelet-derived growth factor receptor: Evidence for more than one receptor class. Proc. Natl. Acad. Sci. USA 85: 3435-3439, 1988.
132. Claesson-Welsh L, Eriksson A, Westermark B, Heldin C-H. cDNA cloning and expression of the human A-type PDGF receptor establishes structural similarity to the B-type receptor. Proc. Natl. Acad. Sci. USA 86: 4917-4921, 1989.
133. Sorkin A, Westermark B, Heldin C-H, Claesson-Welsh L. Effect of receptor kinase inactivation on the rate of internalization and degradation of PDGF and the PDGF ß-receptor. J. Cell Biol. 112: 469-478, 1991.
134. Yarden Y, Schlessinger J. Epidermal growth factor induces rapid, reversible aggregation of the purified epidermal growth factor receptor. Biochemistry 26: 1443-1451, 1987.
135. Heldin C-H, Ernlund A, Rorsman C, Rönnstrand L. Dimerization of B type PDGF receptors occurs after ligand binding and is closely associated with receptor kinase activation. J. Biol. Chem. 264: 8905-8912, 1989.
136. Kelly JD, Haldeman BA, Grant FJ, Murray MJ, Seifert RA, Bowen-Pope DF, Cooper JA, Kazlauskas A. Platelet-derived growth factor (PDGF) stimulates PDGF receptor subunit dimerization and intersubunit trans-phosphorylation. J. Biol. Chem. 266: 8987-8992, 1991.
137. Ueno H, Colbert H, Escobedo JA, Williams LT. Inhibition of PDGF ß-receptor signal transduction by coexpression of a truncated receptor. Science 252: 844-848, 1991.

138. Seifert RA, Hart CE, Phillips PE, Forstrom JW, Ross R, Murray MJ, Bowen-Pope DF. Two different subunits associate to create isoform-specific platelet-derived growth factor receptors. J. Biol. Chem. 264: 8771-8778, 1989.
139. Drozdoff V, Pledger WJ. Cellular response to platelet-derived growth factor (PDGF)-AB after downregulation of PDGF α-receptors. J. Biol. Chem. 266: 17165-17172, 1991.
140. Grotendorst GR, Igarashi A, Larson R, Soma Y, Charette M. Differential binding, biological and biochemical actions of recombinant PDGF AA, AB, and BB molecules on connective tissue cells. J. Cell. Physiol. 149: 235-243, 1991.
141. Inui H, Kitami Y, Kondo T, Inagami T. Transduction of mitogenic activity of platelet-derived growth factor (PDGF) AB by PDGF-ß receptor without participation of PDGF-α receptor in vascular smooth muscle cells. J. Biol. Chem. 268: 17045-17050, 1993.
142. Van Zoelen EJJ, Van Rotterdam W, Van de Wetering RAC, Heldin C-H. Differential effects of PDGF isoforms on proliferation of normal rat kidney cells. Growth Factors 9: 329-339, 1993.
143. Ferns GAA, Sprugel KH, Seifert RA, Bowen-Pope DF, Kelly JD, Murray M, Raines EW, Ross R. Relative platelet-derived growth factor receptor subunit expression determines cell migration to different dimeric forms of PDGF. Growth Factors 3: 315-324, 1990.
144. Heidaran MA, Pierce JH, Yu J-C, Lombardi D, Artrip JE, Fleming TP, Thomason A, Aaronson SA. Role of αß receptor heterodimer formation in ß platelet-derived growth factor (PDGF) receptor activation by PDGF-AB. J. Biol. Chem. 266: 20232-20237, 1991.
145. Seifert RA, Van Koppen A, Bowen-Pope DF. PDGF-AB requires PDGF receptor α-subunits for high-affinity, but not for low-affinity, binding and signal transduction. J. Biol. Chem. 268: 4473-4480, 1993.
146. Giese N, LaRochelle WJ, May-Siroff M, Robbins KC, Aaronson SA. A small *v-sis*/platelet-derived growth factor (PDGF) B-protein domain in which subtle conformational changes abrogate PDGF receptor interaction and transforming activity. Mol. Cell. Biol. 10: 5496-5501, 1990.
147. Clements JC, Bawden LJ, Bloxidge RE, Catlin G, Cook AL, Craig S, Drummond AH, Edwards RM, Fallon A, Green DR, Hellewell PG, Kirwin PM, Nayee PD, Richardson SJ, Brown D, Chahwala SB, Snarey M, Winslow D. Two PDGF B-chain residues, arginine 27 and isoleucine 30, mediate receptor binding and activation. EMBO J. 10; 4113-4120, 1991.
148. Cook AL, Kirwin PM, Craig S, Bawden LJ, Green DR, Price MJ, Richardson SJ, Fallon A, Drummond AH, Edwards RM, Clements JM. Purification and analysis of proteinase-resistant mutants of recombinant platelet-derived growth factor-BB exhibiting improved biological activity. Biochem. J. 281: 57-65, 1992.
149. Jaumann M, Tatje D, Hoppe J. Identification of individual amino acids in platelet-derived growth factor that contribute to the specificity towards the ß-type receptor. FEBS Lett. 302: 265-268, 1992.
150. LaRochelle WJ, Giese N, May-Siroff M, Robbins KC, Aaronson SA. Molecular localization of the transforming and secretory properties of PDGF A and PDGF B. Science 248: 1541-1544, 1990.
151. LaRochelle WJ, Pierce JH, May-Siroff M, Giese N, Aaronson SA. Five PDGF B amino acid substitutions convert PDGF A to a PDGF B-like transforming molecule. J. Biol. Chem. 267: 17074-17077, 1992.
152. Fenstermaker RA, Poptie E, Bonfield TL, Knauss TC, Corsillo L, Piskurich JF, Kaetzel CS, Jentoft JE, Gelfland C, DiCorletto PE, Kaetzel DM Jr. A cationic region of the platelet-derived growth factor (PDGF) A-chain (Arg^{159}-Lys^{160}-Lys^{161}) is required for receptor binding and mitogenic activity of the PDGF-AA homodimer. J. Biol. Chem. 268: 10482-10489, 1993.
153. Heidaran MA, Yu J-C, Jensen RA, Pierce JH, Aaronson SA. A deletion in the extracellular domain of the α platelet-derived growth factor (PDGF) receptor differentially impairs PDGF-AA and PDGF-BB binding affinities. J. Biol. Chem. 267: 2884-2887, 1992.
154. Heidaran M, Mahadevan D, LaRochelle WJ. ß PDGFR-IgG chimera demonstrates that human ß PDGFR Ig-like domains 1 to 3 are sufficient for high affinity PDGF BB binding. FASEB J. 9: 140-145, 1995.
155. Yu J-C, Mahadevan D, LaRochelle WJ, Pierce JH, Heidaran MA. Structural coincidence of αPDGFR epitopes to platelet-derived growth factor-AA and a potent neutralizing monoclonal antibody. J. Biol. Chem. 269: 10668-10674, 1994.
156. Rooney BC, Hosang M, Hunziker W. Production of platelet-derived growth factor receptor (PDGFR-ß) in *E. coli*. Mapping ligand binding domain. FEBS Lett. 339: 181-184, 1994.
157. Clarke MF, Westin E, Schmidt D, Josephs SF, Ratner L, Wong-Staal F, Gallo RC, Reitz MSJ. Transformation of NIH 3T3 cells by a human *c-sis* cDNA clone. Nature 308: 464-467, 1984.
158. Gazit A, Igarashi H, Chiu I-M, Srinivasan A, Yaniv A, Tronick SR, Robbins KC, Aaronson SA. Expression of the normal human *sis*/PDGF-2 coding sequence induces cellular transformation. Cell 39: 89-97, 1984.

159. Beckman MP, Betsholtz C, Heldin C-H, Westermark B, DiMarco E, DiFiore PP, Robbins KC, Aaronson SA. Comparison of biological properties and transforming potential of human PDGF-A and PDGF-B chains. Science 241: 1346-1349, 1988.
160. Bejcek BE, Li DY, Deuel TF. Transformation by v-sis occurs by an internal autoactivation mechanism. Science 245; 1496-1499, 1989.
161. Hannink M, Donoghue DJ. Autocrine stimulation by the *v-sis* gene product requires a ligand-receptor interaction at the cell surface. J. Cell Biol. 107: 287-298, 1988.
162. Hart KC, Xu Y-F, Meyer AN, Lee BA, Donoghue DJ. The v-*sis* oncoprotein loses transforming activity when targeted to the early Golgi complex. J. Cell Biol. 127: 1843-1857, 1994.
163. Westermark B, Heldin C-H. Platelet-derived growth factor in autocrine transformation. Cancer Res. 51; 5087-5092, 1991.
164. Valgeirsdottir S, Eriksson A, Nistér M, Heldin C-H, Westermark B, Claesson-Welsh L. Compartmentalization of autocrine signal transduction pathways in sis-transformed NIH 3T3 cells. J. Biol. Chem. 270: 10161-10170, 1995.
165. Sorkin A, Eriksson A, Heldin C-H, Westermark B, Claesson-Welsh L. Pool of ligand-bound platelet-derived growth factor ß-receptors remain activated and tyrosine phosphorylated after internalization. J. Cell. Physiol. 156: 373-382, 1993.
166. Kazlauskas A, Cooper JA. Autophosphorylation of the PDGF receptor in the kinase insert region regulates interactions with cell proteins. Cell 58: 1121-1133, 1989.
167. Kazlauskas A, Durden DL, Cooper JA. Functions of the major tyrosine phosphorylation site of the PDGF receptor ß-subunit. Cell Regul. 2: 413-425, 1991.
168. Eriksson A, Siegbahn A, Westermark B, Heldin C-H, Claesson-Welsh L. PDGF α- and ß-receptors activate unique and common signal transduction pathways. EMBO J. 11: 543-550, 1992.
169. Valius M, Kazlauskas A. Phospholipase Cγ1 and phophatidylinositol 3 kinase are the downstream mediators of the PDGF receptor's mitogenic signal. Cell 73: 321-334, 1993.
170. Rönnstrand L, Mori S Arvidsson AK, Eriksson A, Wernstedt C, Hellman U, Claesson-Welsh C, Heldin C-H. Identification of two C-terminal autophosphorylation sites in the PDGF ß-receptor; involvement in the interaction with phospholipase Cγ. EMBO J. 11: 3911-3919, 1992.
171. Twamley-Stein GM, Pepperkok R, Ansorge W, Courtneidge SA. The Src family tyrosine kinases are required for platelet-derived growth factor-mediated signal transduction in NIH 3T3 cells. Proc. Natl. Acad. Sci. USA 90: 7696-7700, 1993.
172. Benjamin CW, Jones DA. Platelet-derived growth factor stimulates growth factor receptor binding protein-2 association with Shc in vascular smooth muscle cells. J. Biol. Chem. 269: 30911-30916, 1994.
173. Yokote K, Mori S, Hansen K, McGlade J, Pawson T, Heldin C-H, Claesson-Welsh L. Direct interaction between Shc and the platelet-derived growth factor ß-receptor. J. Biol. Chem. 269: 15337-15343, 1994.
174. Li W, Nishimura R, Kashishian A, Batzer AG, Kim WJH, Cooper JA, Schlessinger J. A new function for a phosphotyrosine phosphatase: linking GRB2-Sos to a receptor tyrosine kinase. Mol. Cell. Biol. 14: 509-517, 1994.
175. Arvidsson AK, Rupp E, Nånberg E, Downward J, Rönnstrand L, Wennström S, Schlessinger J, Heldin C-H, Claesson-Welsh L. Tyr-716 in the platelet-derived growth factor ß-receptor kinase insert is involved in GRB2 binding and Ras activation. Mol. Cell. Biol. 14, 6715-6726, 1994.
176. Ming X-F, Burgering BMTh, Wennström S, Claesson-Welsh L, Heldin C-H, Bos JL, Kozma SC, Thomas G. Activation of p70/p85 S6 kinase by a pathway independent of $p21^{ras}$. Nature 371: 426-429, 1994.
177. Arvidsson AK, Heldin C-H, Claesson-Welsh L. Transduction of circular membrane ruffling by the platelet-derived growth factor ß-receptor is dependent on its kinase insert. Cell Growth Differ. 3: 881-887, 1992.
178. Wennström S, Landgren E, Blume-Jensen P, Claesson-Welsh L. The platelet-derived growth factor ß-receptor kinase insert confers specific signaling properties to a chimeric fibroblast growth factor receptor. J. Biol. Chem. 267: 13749-13756, 1992.
179. Wennström S, Siegbahn A, Yokote K, Arvidsson AK, Heldin C-H, Mori S, Claesson-Welsh L. Membrane ruffling and chemotaxis transduced by the PDGF ß-receptor require the binding site for phosphatidylinositol 3' kinase. Oncogene 9: 651-660, 1994.
180. Chen H-C, Guan J-L. Stimulation of phosphatidylinositol 3'-kinase association with focal adhesion kinase by platelet-derived growth factor. J. Biol. chem. 269: 31229-31233, 1994.
181. Kundra V, Escobedo JA, Kazlauskas A, Kim HK, Rhee SG, Williams LT, Zetter BR. Regulation of chemotaxis by the platelet-derived growth factor receptor-ß. Nature 367: 474-476, 1994.
182. Heidaran MA, Beeler JF, Yu J-C, Ishibashi T, LaRochelle WJ, Pierce JH, Aaronson SA. Differences in substrate specificities of α and ß platelet-derived growth factor (PDGF) receptors. J. Biol. Chem. 268: 9287-9295, 1993.

183. Inui H, Kitami Y, Tani M, Kondo T, Inagami T. Differences in signal transduction between platelet-derived growth factor (PDGF) α and ß receptors in vascular smooth muscle cells. J. Biol. Chem. 269: 30546-30552, 1994.
184. Rupp E, Siegbahn A, Rönnstrand L, Wernstedt C, Claesson-Welsh L, Heldin C-H. A unique autophosphorylation site in the platelet-derived growth factor α receptor from a heterodimeric receptor complex. Eur. J. Biochem. 225: 29-41, 1994.
185. Cochran BH, Reffel AC, Stiles CD. Molecular cloning of gene sequences regulated by platelet-derived growth factor. Cell 33: 939-947, 1983.
186. Linzer DIH, Nathans D. Growth-related changes in specific mRNAs of cultured mouse cells. Proc. Natl. Acad. Sci. USA 80: 4271-4275, 1983.
187. Ransone LJ, Verma IM. Nuclear proto-oncogenes *fos* and *jun*. Annu. Rev. Cell Biol. 6: 539-557, 1990.
188. Salhany KE, Robinson-Benion C, Candia AF, Pledger WJ, Holt JT. Differential induction of the c-fos promoter through distinct PDGF receptor signaling pathways. J. Cell. Physiol. 150: 386-395, 1992.
189. Jackson J, Holt JT, Pledger WJ. Platelet-derived growth factor regulation of Fos stability correlates with growth induction. J. Biol. Chem. 267: 17444-17448, 1992.
190. Surmacz E, Reiss K, Sell C, Baserga R. Cyclin D1 messenger RNA is inducible by platelet-derived growth factor in cultured fibroblasts. Cancer Res. 52: 4522-4525, 1992.
191. Winston JT, Pledger WJ. Growth factor regulation of cyclin D1 mRNA expression through protein synthesis-dependent and -independent mechanisms. Mol. Biol. Cell 4: 1133-1144, 1993.
192. Mori S, Rönnstrand L, Claesson-Welsh L, Heldin C-H. A tyrosine residue in the juxtamembrane segment of the platelet-derived growth factor ß-receptor is critical for ligand-mediated endocytosis. J. Biol. Chem. 269: 4917-4921, 1994.
193. Joly M, Kazlauskas A, Fay FS, Corvera S. Disruption of PDGF receptor trafficking by mutation of its PI-3 kinase binding sites. Science 263: 684-687, 1994.
194. Mori S, Heldin C-H, Claesson-Welsh L. Ligand-induced ubiquitination of the platelet-derived growth factor ß-receptor plays a negative regulatory role in its mitogenic signaling. J. Biol. Chem. 268: 577-583, 1993.
195. Goustin AS, Betsholtz C, Pfeifer-Ohlsson S, Persson H, Rydnert J, Bywater M, Holmgren G, Heldin C-H, Westermark B, Ohlsson R. Coexpression of the *sis* and *myc* proto-oncogenes in developing human placenta suggests autocrine control of trophoblast growth. Cell 41: 301-312, 1985.
196. Holmgren L, Claesson-Welsh L, Heldin C-H, Ohlsson R. The expression of PDGF α- and ß-receptors in subpopulations of PDGF-producing cells implicates autocrine stimulatory loops in the control of proliferation in cytotrophoblasts that have induced the maternal endometrium. Growth Factors 6: 219-232, 1992.
197. Rappolee DA, Brenner CA, Schultz R, Mark D, Werb Z. Developmental expression of PDGF, TGF-α, and TGF-ß genes in preimplantation mouse embryos. Science 241: 1823-1825, 1988.
198. Richardson WD, Pringle N, Mosley MJ, Western B, Dubois-Dalcq M. A role for platelet-derived growth factor in normal gliogenesis in the central nervous system. Cell 53: 309-319, 1988.
199. Mercola M, Wang C, Kelly J, Brownlee C, Jackson-Grusby L, Stiles C, Bowen-Pope D. Selective expression of PDGF A and its receptor during early mouse embryogenesis. Dev. Biol. 138: 114-123, 1990.
200. Soriano P. Abnormal kidney development and hematological disorders in PDGF ß-receptor mutant mice. Gene Dev. 8: 1888-1896, 1994.
201. Levéen P, Pekny M, Gebre-Medhin S, Swolin B, Larsson E, Betsholtz C. Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. Gene Dev. 8: 1875-1887, 1994.
202. Schatteman GC, Morrison-Graham K, Van Koppen A, Weston JA, Bowen-Pope DF. Regulation and role of PDGF receptor α-subunit expression during embryogenesis. Development 115: 123-131, 1992.
203. Palmieri SL, Payne J, Stiles CD, Biggers JD, Mercola M. Expression of PDGF-A and PDGF-α receptor genes during pre- and post-implantation development: evidence for a developmental shift from an autocrine to a paracrine mode of action. Mech. Develop. 39: 181-191, 1992.
204. Stephenson DA, Mercola M, Andersen E, Wang C-J, Stiles CD, Bowen-Pope DF, Chapman VM. Platelet-derived growth factor receptor α-subunit gene (*Pdgfra*) is deleted in the mouse patch (Ph) mutation. Proc. Natl. Acad. Sci. USA 88: 6-10, 1991.
205. Pringle NG, Richardson WD. A singularity of PDGF α-receptor expression in the dorsoventral axis of the neural tube may define the origin of the oligodendrocyte lineage. Development 117: 525-533, 1993.
206. Yeh H-J, Silos-Santiago I, Wang Y-X, George RJ, Snider WD, Deuel TF. Developmental expression of the platelet-derived growth factor α-receptor gene in mammalian central nervous system. Proc. Natl. Acad. Sci. USA 90: 1952-1956, 1993.
207. Smits A, Kato M, Westermark B, Nistér M, Heldin C-H, Funa K. Neurotrophic activity of platelet-derived growth factor (PDGF): rat neuronal cells possess functional PDGF ß-type receptors and respond to PDGF. Proc. Natl. Acad. Sci. USA 88: 8159-8163, 1991.

208. Eccleston PA, Funa K, Heldin C-H. Expression of platelet-derived growth factor (PDGF) and PDGF α- and ß-receptors in the peripheral nervous system: an analysis of sciatic nerve and dorsal root ganglia. Dev. Biol. 155: 459-470, 1993.
209. DiCorleto PE, Bowen-Pope DF. Cultured endothelial cells produce a platelet-derived growth factor-like protein. Proc. Natl. Acad. Sci. USA 80: 1919-1923, 1983.
210. Collins T, Ginsburg D, Boss JM, Orkin SH, Pober JS. Cultured human endothelial cells express platelet-derived growth factor B chain: cDNA cloning and structural analysis. Nature 316: 748-750, 1985.
211. Collins T, Pober JS, Gimbrone MA Jr, Hammacher A, Betsholtz C, Westermark B, Heldin C-H. Cultured human endothelial cells express platelet-derived growth factor A chain. Am. J. Pathol. 126: 7-12, 1987.
212. Koyama N, Watanabe S, Tezuka M, Morisaki N, Saito Y, Yoshida S. Migratory and proliferative effect of platelet-derived growth factor in rabbit retinal endothelial cells: evidence of an autocrine pathway of platelet-derived growth factor. J. Cell. Physiol. 158: 1-6, 1994.
213. Seifert RA, Schwartz SM, Bowen-Pope DF. Developmentally regulated production of platelet-derived growth factor-like molecules. Nature 311, 669-671, 1984.
214. Nilsson J, Sjölund M, Palmberg L, Thyberg J, Heldin C-H. Arterial smooth muscle cells in primary culture produce a platelet-derived growth factor-like protein. Proc. Natl. Acad. Sci. USA 82: 4418-4422, 1985.
215. Ansel JC, Tiesman JP, Olerud JE, Krueger JG, Krane JF, Tara DC, Shipley GD, Gilbertson D, Usui ML, Hart CE. Human keratinocytes are a major source of cutaneous platelet-derived growth factor. J. Clin. Invest. 92: 671-678, 1993.
216. Vignaud JM, Allam M, Martinet N, Pech M, Plenat F, Martinet Y. Presence of platelet-derived growth factor in normal and fibrotic lung is specifically associated with interstitial macrophages, while both interstitial and alveolar epithelial cells express the *c-sis* proto-oncogene. Am. J. Resp. Cell Mol. Biol. 5: 531-538, 1991.
217. Shimokado K, Raines EW, Madtes DK, Barrett TB, Benditt EP, Ross R. A significant part of macrophage-derived growth factor consists of at least two forms of PDGF. Cell 43: 277-286, 1985.
218. Martinet Y, Bitterman PB, Mornex J-F, Grotendorst GR, Martin GR, Crystal RG. Activated human monocytes express the *c-sis* proto-oncogene and release a mediator showing PDGF-like activity. Nature 319: 158-160, 1986.
219. Martinet Y, Rom WN, Grotendorst GR, Martin GR, Crystal RG. Exaggerated spontaneous release of platelet-derived growth factor by alveolar macrophages from patients with idiopathic pulmonary fibrosis. N. Engl. J. Med. 317: 202-209, 1987.
220. Heldin C-H, Hammacher A, Nistér M, Westermark B. Structural and functional aspects of platelet-derived growth factor. Br. J. Cancer 57: 591-593, 1988.
221. Deuel TF, Senior RM, Huang JS, Griffin GL. Chemotaxis of monocytes and neutrophils to platelet-derived growth factor. J. Clin. Invest. 69: 1046-1049, 1982.
222. Shure D, Senior RM, Griffin GL, Deuel TF. PDGF AA homodimers are potent chemoattractants for fibroblasts and neutrophils, and for monocytes activated by lymphocytes or cytokines. Biochem. Biophys. Res. Comm. 186: 1510-1514, 1992.
223. Bar RS, Boes M, Booth BA, Dake BL, Henley S, Hart MN. The effects of platelet-derived growth factor in cultured microvessel endothelial cells. Endocrinology 124: 1841-1848, 1989.
224. Smits A, Hermanson M, Nistér M, Karnushina I, Heldin C-H, Westermark B, Funa K. Rat brain capillary endothelial cells express functional PDGF B-type receptors. Growth Factors 2: 1-8, 1989.
225. Reuterdahl C, Sundberg C, Rubin K, Funa K, Gerdin B. Tissue localization of ß receptors for platelet-derived growth factor and platelet-derived growth factor B chain during wound repair in humans. J. Clin. Invest. 91: 2065-2075, 1993.
226. Risau W, Drexler H, Mironov V, Smits A, Siegbahn A, Funa K, Heldin C-H. Platelet-derived growth factor is angiogenic *in vivo*. Growth Factors, 1992.
227. Robson MC, Phillips LG, Thomason A, Robson LE, Pierce GF. Platelet-derived growth factor BB for the treatment of chronic pressure ulcers. Lancet 339: 23-25, 1992.
228. Pierce GF, Tarpley JE, Allman RM, Goode PS, Serdar CM, Morris B, Mustoe TA, VandeBerg J. Tissue repair processes in healing chronic pressure ulcers treated with recombinant platelet-derived growth factor BB. Am. J. Pathol. 145: 1399-1410, 1994.
229. Bonner JC, Osornio-Vargas AR, Badgett A, Brody A. Differential proliferation of rat lung fibroblasts induced by the platelet-derived growth factor-AA, and -BB isoforms secreted by rat alveolar macrophages. Am. J. Resp. Cell Mol. Biol. 5: 539-547, 1991.
230. Ross R. The pathogenesis of atherosclerosis: an update. N. Engl. J. Med. 314: 488-500, 1986.
231. Ferns GAA, Raines EW, Sprugel KH, Motani AS, Reidy MA, Ross R. Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. Science 253; 1129-1132, 1991.

232. Reuterdahl C, Tingström A, Terracio, Funa K, Heldin C-H, Rubin K. Characterization of platelet-derived growth factor ß-receptor expressing cells in the vasculature of human rheumatoid synovium. Lab. Invest. 64; 321-329, 1991.
233. Fleming TP, Matsui T, Heidaran MA, Molloy CJ, Artrip J, Aaronson SA. Demonstration of an activated platelet-derived growth factor autocrine pathway and its role in human tumour cell proliferation *in vitro*. Oncogene 7: 1355-1359, 1992.
234. Hermanson M, Funa K, Hartman M, Claesson-Welsh L, Heldin C-H , Westermark B, Nistér M. Platelet-derived growth factor and its receptors in human glioma tissue: expression of messenger RNA and protein suggests the presence of autocrine and paracrine loops. Cancer Res. 52: 3213-3219, 1992.
235. Plate KH, Breier G, Farrell CL, Risau W. Platelet-derived growth factor receptor-ß is induced during tumor development and upregulated during tumor progression in endothelial cells in human gliomas. Lab. Invest. 67: 529-534, 1992.
236. Forsberg K, Valyi-Nagy I, Heldin C-H, Herlyn M, Westermark B. Platelet-derived growth factor (PDGF) in oncogenesis; development of a vascular connective tissue stroma in xenotransplanted human melanoma producing PDGF-BB. Proc. Natl. Acad. Sci. USA 90: 393-397, 1993.
237. Vassbotn F, Östman A, Siegbahn A, Holmsen H, Heldin C-H. Neomycin is a platelet-derived growth factor (PDGF) antagonist that allows discrimination of PDGF α- and ß-receptor signals in cells expressing both receptor types. J. Biol. Chem. 267: 15635-15641, 1992.
238. Kovalenko M, Gazit A, Böhmer A, Rorsman C, Rönnstrand L, Heldin C-H, Waltenberger J, Böhmer F-D, Levitzki A. Selective platelet-derived growth factor receptor kinase blockers reverse *sis*-transformation. Cancer Res. 54: 6106-6114, 1994.
239. Engström U, Engström A, Ernlund A, Westermark B, Heldin C-H. Identification of a peptide for platelet-derived growth factor. J. Biol. Chem. 267: 16581-16587, 1992.
240. Ueno H, Escobedo JA, Williams LT. Dominant-negative mutations of platelet-derived growth factor (PDGF) receptors. J. Biol. Chem. 268: 22814-22819, 1993.
241. Kemp PR, Shachar-Hill Y, Weissberg PL, Metcalfe JC. Inhibition of PDGF BB stimulated DNA synthesis in rat aortic vascular smooth muscle cells by the expression of a truncated PDGFß receptor. FEBS Lett. 336: 119-123, 1993.
242. Tiesman J, Hart CE. Identification of a soluble receptor for platelet-derived growth factor in cell-conditioned medium and human plasma. J. Biol. Chem. 268: 9621-9628, 1993.
243. Vassbotn F, Östman A, Langeland N, Holmsen H, Westermark B, Heldin C-H, Nistér M. Activated platelet-derived growth factor autocrine pathway drives the transformed phenotype of a human glioblastoma cell line. J. Cell. Physiol. 158: 381-389, 1994.
244. LaRochelle WJ, Jensen RA, Heidaran MA, May-Siroff M, Wang LM, Aaronson SA, Pierce JH. Inhibition of platelet-derived growth factor autocrine growth stimulation by a monoclonal antibody to the human α platelet-derived growth factor receptor. Cell Growth Differ. 4: 547-553, 1993.
245. Vassbotn F, Andersson M, Westermark B, Heldin C-H, Östman A. Reversion of autocrine transformation by a dominant negative platelet-derived growth factor mutant. Mol. Cell. Biol. 13: 4066-4076, 1993.
246. Shamah SM, Stiles CD, Guha A. Dominant-negative mutants of platelet-derived growth factor revert the transformed phenotype of human astrocytoma cells. Mol. Cell. Biol. 13: 7203-7212, 1993.
247. Mori S, Rönnstrand L, Yokote K, Engström A, Courtneidge SA, Claesson-Welsh L, Heldin C-H. Identification of two juxtamembrane autophosphorylation sites in the PDGF ß-receptor; involvement in the interaction with Src family tyrosine kinases. EMBO J. 12: 2257-2264, 1993.
248. Kashishian A, Kazlauskas A, Cooper JA. Phosphorylation sites in the PDGF receptor with different specificities for binding phospholipase GAP and PI3 kinase *in vivo*. EMBO J. 11: 1373-1382, 1992.
249. Panayotou G, Bax B, Gout I, Federwisch M, Wroblowski B, Dhand R, Fry MJ, Blundell TL, Wollmer A, Waterfield MD. Interaction of the p85 subunit of PI3 kinase and its N-terminal SH2 domain with a PDGF receptor phosphorylation site: structural features and analysis of conformational changes. EMBO J. 11: 4261-4272, 1992.
250. Nishimura R, Li W, Kashishian A, Mondino A, Cooper J, Schlessinger J. Two signaling molecules share a phosphotyrosine-containing binding site in the platelet-derived growth factor receptor. Mol. Cell. Biol. 13: 6889-6896, 1993.
251. Fantl WJ, Escobedo JA, Martin GA, Tuck CW, DelRosario M, McCormick F, Williams LT. Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signaling pathways. Cell 69: 413-423, 1992.
252. Kashishian A, Cooper JA. Phosphorylation sites at the C-terminus of the platelet-derived growth factor receptor bind phospholipase Cγ1. Mol. Biol. Cell 4: 49-57, 1993.

253. Valius M, Bazenet C, Kazlauskas A. Tyrosines 1021 and 1009 are phosphorylation sites in the carboxy terminus of the platelet-derived growth factor receptor ß-subunit and are required for binding of phopholipase Cγ and a 64 kilodalton protein, respectively. Mol. Cell. Biol. 13: 133-143, 1993.
254. Larose L, Gish G, Shoelson S, Pawson T. Identification of residues in the ß platelet-derived growth factor receptor that confer specificity for binding to phospholipase Cγ1. Oncogene 8: 2493-2499, 1993.
255. Kazlauskas A, Feng G-S, Pawson T, Valius M. The 64 kDa protein that associates with the platelet-derived growth factor receptor ß subunit via Tyr 1009 is the SH2-containing phophotyrosine phosphatase Syp. Proc. Natl. Acad. Sci. USA 90: 6939-6942, 1993.
256. Lechleider RJ, Sugimoto S, Bennett AM, Kashishian A, Cooper JA, Shoelson SE, Walsh CT, Neel BG. Activation of the SH2-containing phosphotyrosine phosphatase SH-PTP2 by its binding site, phosphotyrosine 1009, on the human platelet-derived growth factor receptor ß. J. Biol. Chem. 268; 21478-21481, 1993.
257. Nistér M, Libermann TA, Betsholtz C, Pettersson M, Claesson-Welsh L, Heldin C-H, Schlessinger J, Westermark B. Expression of messenger RNAs for platelet-derived growth factor and transforming growth factor-α and their receptors in human malignant glioma cell lines. Cancer Res. 48: 3910-3918, 1988.
258. Nistér M, Claesson-Welsh L, Eriksson A, Heldin C-H, Westermark B. Differential expression of platelet-derived growth factor receptors in human malignant glioma cell lines. J. Biol. Chem. 266: 16755-16763, 1991.
259. Söderdahl G, Betsholtz C, Johansson A, Nilsson K, Bergh J. Differential expression of platelet-derived growth factor and transforming growth factor genes in small- and non-small-cell human lung carcinoma lines. Int. J. Cancer 41: 636-641, 1988.
260. Forsberg K, Bergh J, Westermark B. Expression of functional PDGF ß receptors in a human large cell lung-carcinoma cell line. Int. J. Cancer 53: 556-560, 1993.
261. Gerwin BI, Lechner JF, Reddel RR, Roberts AB, Robbins KC, Gabrielson EW, Harris CC. Comparison of production of transforming growth factor-β and platelet-derived growth factor by normal human mesothelial cells and mesothelioma cell lines. Cancer Res. 47: 6180-6184, 1987.
262. Versnel MA, Hagemeijer A, Bouts MJ, Van der Kwast ThH, Hoogsteden HC. Expression of c-sis (PDGF B-chain) and PDGF A-chain genes in ten human malignant mesothelioma cell lines derived from primary and metastatic tumors. Oncogene 2: 601-605, 1988.
263. Versnel MA, Claesson-Welsh L, Hammacher A, Bouts MJ, Van der Kwast ThH, Eriksson A, Willemsen R, Weima SM, Hoogsteden HC, Hagemeijer A, Heldin C-H. Human malignant mesothelioma cell lines express PDGF β-receptors whereas cultured normal mesothelial cells express predominantly PDGF α-receptors. Oncogene 6: 2005-2011, 1991.
264. Perez R, Betsholtz C, Westermark B, Heldin C-H. Frequent expression of growth factors for mesenchymal cells in human mammary carcinoma cell lines. Cancer Res. 47: 3425-3429, 1987.
265. Albino AP, Davis BM, Nanus DM. Induction of growth factor RNA expression in human malignant melanoma: markers of transformation. Cancer Res. 51: 4815-4820, 1991.
266. Sitaras NM, Sariban E, Bravo M, Pantazis P, Antoniades HN. Constitutive production of platelet-derived growth factor-like proteins by human prostate carcinoma cell lines. Cancer Res. 48: 1930-1935, 1988.
267. Levéen P, Claesson-Welsh L, Heldin C-H, Westermark B, Betsholtz C. Expression of messenger RNAs for platelet-derived growth factor and its receptors in human sarcoma cell lines. Int. J. Cancer 46: 1066-1070, 1990.
268. Heldin NE, Cvejic D, Smeds S, Westermark B. Coexpression of functionally active receptors for thyrotropin and platelet-derived growth factor in human thyroid carcinoma cells. Endocrinology 129: 2187-2193, 1991.
269. Pontén F, Ren Z, Nistér M, Westermark B, Pontén J. Epithelial-stromal interactions in basal cell cancer: the PDGF system. J. Invest. Dermatol. 102: 304-309, 1994.
270. Holmgren L, Flam F, Larsson E, Ohlsson R. Succesive activation of the platelet-derived growth factor ß receptor and platelet-derived growth factor B genes correlates with the genesis of human choriocarcinoma. Cancer Res. 53: 2927-2931, 1993.
271. Lindmark G, Sundberg C, Glimelius B, Pahlman L, Rubin K, Gerdin B. Stromal expression of platelet-derived growth factor ß-receptor and platelet-derived growth factor B-chain in colorectal cancer. Lab. Invest. 69: 682-689, 1993.
272. Smits A, Funa K, Vassbotn FS, Beausang-Linder M, af Ekenstam F, Heldin C-H, Westermark B, Nistér M. Expression of platelet-derived growth factor and its receptors in proliferative disorders of fibroblastic origin. Am. J. Pathol. 140: 639-648, 1992.
273. Fleming TP, Saxena A, Clark WC, Robertson JT, Oldfield EH, Aaronson SA, Ali IU. Amplification and/or overexpression of platelet-derived growth factor receptors and epidermal growth factor receptor in human glial tumors. Cancer Res. 52: 4550-4553, 1992.

274. Antoniades HN, Galanopoulos T, Neville-Golden J, O'Hara CJ. Malignant epithelial cells in primary human lung carcinomas coexpress *in vivo* platelet-derived growth factor (PDGF) and PDGF receptor mRNAs and their protein products. Proc. Natl. Acad. Sci. USA 89: 3942-3946, 1992.
275. Henriksen R, Funa K, Wilander E, Bäckström T, Riderheim M, Oberg K. Expression and prognostic significance of platelet-derived growth factor and its receptors in epithelial ovarian neoplasms. Cancer Res. 53: 4550-4554, 1993.
276. Versnel MA, Haarbrink M, Langerak AW, De Laat PAJM, Hagemeijer A, Van der Kwast ThH, Van den Berg-Bakker LAM, Schrier PI. Human ovarian tumors of epithelial origin express PDGF in vitro and in vivo. Cancer Genet. Cytogenet. 73: 60-64, 1994.
277. Wang J, Coltrera MD, Gown AM. Cell proliferation in human soft tissue tumors correlates with platelet-derived growth factor B chain expression: an immunohistochemical and *in situ* hybrization study. Cancer Res. 54: 560564, 1994.
278. Zawel L, Reinberg D. Initiation of transcription by RNA polymerase II: a multi-step process. Prog. Nucleic Acids Res. Mol. Biol. 44: 68-108, 1993.
279. Conaway RC, Conaway JW. General initiation factors for RNA polymerase II. Annu. Rev. Biochem. 62: 161-190, 1993.
280. Buratowsky S. The basics of basal transcription by RNA polymerase II. Cell 77: 1-3, 1994.
281. Pugh BF, Tjian R. Mechanism of transcriptional activation by Sp1: evidence for coactivators. Cell 61: 1187-1197, 1990.
282. Jakobovits EB, Schlokat U, Vannice JL, Derynck R, Levinson AD. The human transforming growth factor alpha promoter directs transcription initiation from a single site in the absence of a TATA sequence. Mol. Cell. Biol. 8: 5549-5554, 1988.
283. Ishii S, Xu YH, Stratton RH, Roe BA, Merlino GT, Pastan I. Characterization of the promoter region of the human epidermal growth factor receptor gene. Proc. Natl. Acad. Sci. USA 82: 4920-4924, 1985.
284. Weis L, Reinberg D. Transcription by RNA polymerase II: initiator-directed formation of transcription-competent complexes. FASEB J. 6: 3300-3309, 1992.
285. Fraizer GC, Wu YJ, Hewitt SM, Maity T, Ton CCT, Huff V, Saunders GF. Transcriptional regulation of the Wilms' tumor gene (WT 1). J. Biol. Chem. 269: 8892-8900, 1994.
286. Godowski PJ, Picard D. Steroid receptors. How to be both a receptor and a transcription factor. Biochem. Pharmacol. 38: 3135-3143, 1989.
287. Bauerle PA, Baltimore D. I kappa B: a specific inhibitor of the NF kappa B transcription factor. Science 242, 540-546, 1988.
288. Bohmann D, Bos TJ, Admon A, Nishimura T, Vogt PK, Tjian R. Human proto-oncogene *c-jun* encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. Science 238: 1386-1392, 1987.
289. Yamamoto KR, Gonzalez GA, Menzel P, Rivier J, Montminy MR. Characterization of a bipartite activation domain in transcription factor CREB. Cell 60: 611-617, 1990.
290. Tjian R, Maniatis T. Transcriptional activation: a complex puzzle with few easy pieces. Cell 77: 5-8, 1994.
291. Thanos D, Maniatis T. The high mobility group protein HMG I(Y) is required for NF-kB-dependent viral induction of the human IFN-ß gene. Cell 71: 777-789, 1992.
292. Herschbach BM, Johnson AD. Transcriptional repression in eukaryotes. Annu. Rev. Cell. Biol. 9: 479-509, 1993.
293. Cowell IG. Repression versus activation in the control of gene transcription. Trends Biochem. Sci. 19: 38-42, 1994.
294. Liu Y, Yang N, Teng CT. COUP-TF acts as a competitive repressor for estrogen receptor-mediated activation of the mouse lactoferrin gene. Mol. Cell. Biol. 13: 1836-1846, 1993.
295. Cahill MA, Ernst WH, Janknecht R, Nordheim A. Regulatory squelching. FEBS Lett. 344: 105-108, 1994.
296. Wolffe AP. Transcription: in tune with the histones. Cell 77: 13-16, 1994.
297. Lee DY, Hayes JJ, Pruss D, Wolffe AP. A positive role for histone acetylation in transcription factor access to nucleosomal DNA. Cell 72: 73-84, 1993.
298. López-Rodas G, Brosch G, Georgevia EI, Sendra R, Franco L, Loidl P. Histone deacetylase. A key enzyme for the binding of regulatory proteins to chromatin. FEBS Lett. 317: 175-180, 1993.
299. Lu Q, Wallrath LL, Granok H, Elgin SCR. $(CT)_n$ $(GA)_n$ repeats and heat shock elements have distinct roles in chromatin structure and transcriptional activation of the Drosophila hsp26 gene. Mol. Cell. Biol. 13: 2802-2814, 1993.
300. Dunaway M, Ostrander EA. Local domains of supercoiling activate a eukaryotic promoter *in vivo*. Nature 361: 746-748, 1993.

301. Stein GS, Lian JB, Dworetzky SI, Owen TA, Bortell R, Bidwell JP, van Wijnen AJ. Regulation of transcription-factor activity during growth and differentiation: involvement of the nuclear matrix in concentration and localization of promoter binding proteins. J. Cell. Biochem. 47: 300-305, 1991.
302. Tycko B. Genomic imprinting: mechanism and role in human pathology. Am. J. Pathol. 144: 431-443, 1994.
303. Sturm R, Das G, Herr W. The ubiquitous octamer binding protein Oct-1 contains a POU domain with a homeobox subdomain. Gene Dev. 2: 1582-1599, 1988.
304. Neuberg M, Adamkiewicz J, Hunter JB, Müller R. A Fos protein containing the Jun leucine zipper forms a homodimer binding to the AP1 site. Nature 341: 243-245, 1989.
305. Turner R, Tjian R. Leucine repeats and an adjacent DNA binding domain mediate the formation of functional cFos-cJun heterodimers. Science 243: 1689-1694, 1989.
306. Voronova A, Baltimore D. Mutations that disrupt DNA binding and dimer formation in the E47 helix-loop-helix protein map to distinct domains. Proc. Natl. Acad. Sci. USA 87: 4722-4726, 1990.
307. Kadonaga JT, Carner KR, Masiarz FR, Tjian R. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell 51: 1079-1090, 1987.
308. Sap J, Munoz A, Schmitt J, Stunnenberg H, Vennström B. Repression of transcription mediated at a thyroid hormone response element by the v-*erb*-A oncogene product. Nature 340: 242-244, 1989.
309. Landsman D, Bustin M. A signature for the HMG-1 box DNA-binding proteins. Bioessays 15: 539-546, 1993.
310. Giese K, Cox J, Grosschedl R. The HMG domain of lymphoid enhancer factor 1 bends DNA and facilitates assembly of functional nucleoprotein structures. Cell 69: 185-196, 1992.
311. Landschulz WH, Johnson PF, McKnight SL. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science 240: 1759-1764, 1988.
312. Gentz R, Rauscher FJ III, Abate C, Curran T. Parallel association of Fos and Jun leucine zippers juxtaposes DNA binding domains. Science 243: 1695-1699, 1989.
313. Murre C, McCaw PC, Baltimore D. A new DNA-binding and dimerization motif in immunoglobulin enhancer binding, *daughterless*, *MyoD*, and *myc* proteins. Cell 56: 777-783, 1989.
314. Hollenberg SM, Evans RM. Multiple and cooperative trans-activation domains of the human glucocoricoid receptor. Cell 55: 899-906, 1988.
315. Madden SL, Cook DM, Morris JF, Gashler A, Sukhatme VP, Rauscher FJ III. Transcriptional repression mediated by the WT 1 Wilms tumor gene product. Science 253: 1550-1553, 1991.
316. Courey AJ, Tjian R. Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. Cell 55: 887-898, 1988.
317. Berk BC, Rao GN. Angiotensin II-induced vascular smooth muscle cell hypertrophy: PDGF A-chain mediates the increase in cell size. J. Cell. Physiol. 154: 368-380, 1993.
318. Zwijsen RML, Japenga SC, Heijen AMP, van den Bos R, Koeman JH. Induction of platelet-derived growth factor chain A gene expression in human smooth muscle cells by oxidized low density lipoproteins. Biochem. Biophys. Res. Comm. 186: 1410-1416, 1992.
319. Kourembanas S, Hannan RL, Faller DV. Oxygen tension regulates the expression of the platelet-derived growth factor B-chain gene in human endothelial cells. J. Clin. Invest. 86: 670-674, 1990.
320. Hsieh H-J, Li N-Q, Frangos JA. Shear-induced platelet-derived growth factor gene expression in human endothelial cells is mediated by protein kinase C. J. Cell. Physiol. 150: 552-558, 1992.
321. Resnick N, Collins T, Atkinson W, Bonthron DT, Forbes Dewey C Jr, Gimbrone MA Jr. Platelet-derived growth factor B chain promoter contains a cis-acting fluid shear-stress-responsive element. Proc. Natl. Acad. Sci. USA 90: 4591-4595, 1993.
322. Terracio L, Rönnstrand L, Tingström A, Rubin K, Claesson-Welsh L, Funa K, Heldin C-H. Induction of platelet-derived growth factor receptor expression in smooth muscle cells and fibroblasts upon tissue culturing. J. Cell Biol. 107: 1947-1957, 1988.
323. Marx M, Daniel TO, Kashgarian M, Madri JA. Spatial organization of the extracellular matrix modulates the expression of PDGF-receptor subunits in mesangial cells. Kidney Int. 43: 1027-1041, 1993.
324. Leof EB, Proper JA, Goustin AS, Shipley GD, DiCorleto PE, Moses HL. Induction of c-*sis* mRNA and activity similar to platelet-derived growth factor by transforming growth factor ß: a proposed model for indirect mitogenesis involving autocrine activity. Proc. Natl. Acad. Sci. USA 83: 2453-2457, 1986.
325. Janat MF, Liau G. Transforming growth factor-ß1 is a powerful modulator of platelet-derived growth factor action in smooth muscle cells. J. Cell. Physiol. 150: 232-242, 1992.
326. Haberstroh U, Zahner G, Disser M, Thaiss F, Wolf G, Stahl RAK. TGF-ß stimulates rat mesangial cell proliferation in culture: role of PDGF ß-receptor expression. Am. J. Physiol. 264: F199-F205, 1993.
327. Soma Y, Grotendorst GR. TGF-ß stimulates primary human skin fibroblast DNA synthesis via an autocrine production of PDGF-related peptides. J. Cell. Physiol. 140: 246-253, 1989.

328. Majack RA, Majesky MW, Goodman LV. Role of PDGF-A expression in the control of vascular smooth muscle cell growth by transforming growth factor-ß. J. Cell Biol. 111; 239-247, 1990.
329. Win KM, Charlotte F, Mallat A, Cherqui D, Martin N, Mavier P, Preaux AM, Dhumeaux D, Rosenbaum J. Mitogenic effect of transforming growth factor-ß1 on human Ito cells in culture: evidence for mediation by platelet-derived growth factor. Hepatology 18: 137-145, 1994.
330. Paulsson Y, Karlsson C, Heldin C-H, Westermark B. Density-dependent inhibitory effect of transforming growth factor-ß1 on human fibroblasts involves the down-regulation of platelet-derived growth factor α-receptors. J. Cell. Physiol. 157: 97-103, 1993.
331. Battegay EJ, Raines EW, Seifert RA, Bowen-Pope DF, Ross R. TGF-ß induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop. Cell 63: 515-524, 1990.
332. Seifert RA, Coats SA, Raines EW, Ross R, Bowen-Pope DF. Platelet-derived growth factor (PDGF) receptor α-subunit mutant and reconstituted cell lines demonstrate that transfroming growth factor-ß can be mitogenic through PDGF A-chain-dependent and -independent pathways. J. Biol. Chem. 269: 13951-13955, 1994.
333. Stouffer GA and Owens GK. TGF-ß promotes proliferation of cultured SMCmes via both PDGF-AA-dependent and PDGF-AA-independent mechanisms. J. Clin. Invest. 93: 2048-2055, 1994.
334. Bronzert DA, Bates SE, Sheridan JP, Lindsey R, Valverius EM, Stampfer MR, Lippman ME, Dickson RB. Transforming growth factor-beta induces platelet-derived growth factor (PDGF) messenger RNA and PDGF secretion while inhibiting growth in normal human mammary epithelial cells. Mol. Endocrinol. 4: 981-989, 1990.
335. Van Zoelen EJJ, Van Rotterdam W, Ward-Van Oostwaard TMJ, Feijen A. Phenotypic transformation of normal rat kidney cells by transforming growth factor ß is not paralleled by enhanced production of a platelet-derived growth factor. Eur. J. Biochem. 209: 89-94, 1992.
336. Kaetzel DM, Coyne DW, Fenstermaker RA. Transcriptional control of the platelet-derived growth factor subunit genes. Biofactors 4: 71-81, 1993.
337. Takimoto Y, Wang ZY, Kobler K, Deuel TF. Promoter region of the human platelet-derived growth factor A-chain gene. Proc. Natl. Acad. Sci. USA 88: 1686-1690, 1991.
338. Lin XH, Wang ZY, Gu LJ, Deuel TF. Functional analysis of the human platelet-derived growth factor A-chain promoter region. J. Biol. Chem. 267: 25614-25619, 1992.
339. Wang ZY, Lin XH, Masaharu N, Qiu QQ, Deuel TF. Binding of single-stranded oligonucleotides to a non-B-form DNA structure results in loss of promoter activity of the platelet-derived growth factor A-chain gene. J. Biol. Chem. 267: 13669-13674, 1992.
340. Wang ZY, Lin XH, Qiu QQ, Deuel TF. Modulation of transcription of the platlet-derived growth factor A-chain gene by a promoter region sensitive to S1 nuclease. J. Biol. Chem. 267: 17022-17031, 1992.
341 Bhandari B, Wenzel UO, Marra F, Abboud HE. A nuclear protein in mesangial cells that binds to the promoter region of the platelet-derived growth factor-A chain gene. J. Biol. Chem. 270: 5541-5548, 1995.
342. Kaetzel DM, Maul RS, Liu B, Bonthron D, Fenstermaker RA, Coyne DW. Platelet-derived growth factor A-chain gene transcription is mediated by positive and negative regulatory regions in the promoter. Biochem. J. 301: 321-327, 1994.
343. Wang ZY, Madden SL, Deuel TF, Rauscher FJ III. The Wilms' tumor gene product WT 1 represses transcription of the platelet-derived growth factor A-chain gene. J. Biol. Chem. 267: 21999-22002, 1992.
344. Wang ZY, Qiu QQ, Enger KT, Deuel TF. A second transcriptionally active DNA-binding site for the Wilms' tumour gene product, WT 1. Proc. Natl. Acad. Sci. USA 90: 8896-8900, 1993.
345. Wang ZY, Deuel TF. An S1 nuclease-sensitive homopurine/homopyrimidine domain in the PDGF A-chain promoter contains a novel binding site for the growth factor-inducible protein EGR-1. Biochem. Biophys. Res. Comm. 188: 433-439, 1992.
346. Wang ZY, Qiu QQ, Deuel TF. An S1 nuclease-sensitive region in the PDGF A-chain promoter contains a positive transcriptional regulatory element. Biochem. Biophys. Res. Comm. 198: 103-110, 1994.
347. Lin XH, Guo C, Gu LJ, Deuel TF. Site-specific methylation inhibits transcriptional activity of platelet-derived growth factor A-chain promoter. J. Biol. Chem. 268: 17334-17340, 1993.
348. Takimoto Y, Kuramoto A. Presence of a regulatory element within the first intron of the human platelet-derived growth factor A-chain gene. Jpn. J. Cancer Res. 84: 1268-1272, 1993.
349. Wang ZY, Masaharu N, Qiu QQ, Deuel TF. An S1 nuclease-sensitive region in the first intron of human platelet-derived growth factor A-chain gene contains a negatively acting cell type-specific regulatory element. Nucleic Acids Res. 22: 457-464, 1994.
350. Takimoto Y, Kuramoto A. Gene regulation by the 5'-untranslated region of the platelet-derived growth factor A-chain. Bioch. Biophys. Acta 1222: 511-514, 1994.

351. Ratner L, Thielan B, Collins T. Sequences of the 5' portion of the human *c-sis* gene: characterization of the transcriptional promoter and regulation of expression of the protein product by 5' untranslated mRNA sequences. Nucleic Acids Res. 15: 6017-6036, 1987.
352. Pech M, Gazit A, Arnstein P, Aaronson SA. Generation of fibrosarcomas *in vivo* by a retrovirus that expresses the normal B-chain of platelet-derived growth factor and mimics the alternative splicing pattern of the *v-sis* oncogene. Proc. Natl. Acad. Sci. USA 86: 2693-2697, 1989.
353. Pech M, Rao CD, Robbins KC, Aaronson SA. Functional identification of regulatory elements within the promoter region of platelet-derived growth factor 2. Mol. Cell. Biol. 9: 396-405, 1989.
354. Jin H-M, Brady ML, Fahl WE. Identification and characterization of an essential, activating regulatory element of the human *SIS/PDGFB* promoter in human megakaryocytes. Proc. Natl. Acad. Sci. USA 90: 7563-7567, 1993.
355. Khachigian LM, Fries JWU, Benz MW, Bonthron DT, Collins T. Novel *cis*-acting elements in the human platelet-derived growth factor B-chain core promoter that mediate gene expression in cultured vascular endothelial cells. J. Biol. Chem. 269: 22647-22656, 1994.
356. Dirks RPH, Jansen HJ, Gerritsma J, Onnekink C, Bloemers HPJ. Localization and functional analysis of DNaseI-hypersensitive sites in the human *c-sis/PDGF-B* gene transcription unit and its flanking regions. Eur. J. Biochem. 211: 509-519, 1993.
357. Jin H-M, Robinson DF, Liang Y, Fahl WE. *SIS/PDGF-B* promoter isolation and charcterization of regulatory elements necessary for basal expression of the *SIS/PDGF-B* gene in U2-OS osteosarcoma cells. J. Biol. Chem. 269: 28648-28654, 1994.
358. Dirks RPH, Jansen HJ, Van Gerven HJ, Onnekink C, Bloemers HPJ. *In vivo* footprinting and functional analysis of the human *c-sis/PDGF-B* gene promoter provides evidence for two binding sites for transcriptional activators. Nucl. Acids Res. 23: 1119-1126, 1995.
359. Ratner L. Regulation of expression of the *c-sis* proto-oncogene. Nucleic Acids Res. 17: 4101-4115, 1989.
360. Franklin GC, Donovan M, Adam GIR, Holmgren L, Pfeifer-Ohlsson S, Ohlsson R. Expression of the human PDGF-B gene is regulated by both positively and negatively acting cell type-specific regulatory elements located in the first intron. EMBO J. 10: 1365-1375, 1991.
361. Dirks RPH, Jansen HJ, Onnekink C, de Jonge RJA, Bloemers HPJ. DNaseI-hypersensitive sites located far upstream of the human *c-sis/PDGF-B* gene comap with transcriptional enhancers and a silencer and are preceded by (part of) a new transcription unit. Eur. J. Biochem. 216: 487-495, 1993.
362. Wang C, Stiles CD. Platelet-derived growth factor α-receptor gene expression: isolation and characterization of the promoter and upstream regulatory elements. Proc. Natl. Acad. Sci. USA 91: 7061-7065, 1994.
363. Afink GB, Nistér M, Stassen BHGJ, Joosten PHLJ, Rademakers PJH, Bongcam-Rudloff E, Van Zoelen EJJ, Mosselman S. Molecular cloning and functional characterization of the human platelet-derived growth factor α-receptor gene promoter. Oncogene, 10: 1667-1672, 1995.
364. Mosselman S, Claesson-Welsh L, Kamphuis JS, Van Zoelen EJJ. Developmentally regulated expression of two novel platelet-derived growth factor α-receptor transcripts in human teratocarcinoma cells. Cancer Res. 54: 220-225, 1994.
365. Eriksson A, Nistér M, Levéen P, Westermark B, Heldin C-H, Claesson-Welsh L. Induction of platelet-derived growth factor α- and ß-receptor mRNA and protein by platelet-derived growth factor BB. J. Biol. Chem. 266: 21138-21144, 1991.
366. Behl C, Bogdahn U, Winkler J, Apfel R, Brysch W, Schlingensiepen K-H. Autoinduction of platelet-derived growth factor (PDGF) A-chain mRNA exxpression in a human malignant melanoma cell line and growth inhibitory effects of PDGF A-chain mRNA-specific antisense molecules. Biochem. Biophys. Res. Comm. 193: 744-751, 1993.
367. Bhandari B, Grandaliano G, Abboud HE. Platelet-derived growth factor (PDGF) BB homodimer regulates PDGF A- and B-chain gene transcription in human mesangial cells. Biochem. J. 297: 385-388, 1994.
368. Starksen NF, Harsh GR IV, Gibbs VC, Williams LT. Regulated expression of the platelet-derived growth factor A-chain gene in microvascular endothelial cells. J. Biol. Chem. 262: 14381-14384, 1987.
369. Daniel TO, Gibbs VC, Milfay DF, Williams LT. Agents that increase cAMP accumulation block endothelial *c-sis* induction by thrombin and transforming growth factor-ß. J. Biol. Chem. 262; 11893-11896, 1987.
370. Paulsson Y, Beckmann MP, Westermark B, Heldin C-H. Density-dependent inhibition of cell growth by transforming growth factor-ß1 in normal human fibroblasts. Growth Factors 1: 19-27, 1988.
371. Yeh YL, Kang YM, Chaibi MS, Xie JF, Graves DT. IL-1 and transforming growth factor-ß inhibit platelet-derived growth factor-AA binding to osteoblastic cells by reducing platelet-derived growth factor-α receptor expression. J. Immunol. 150: 5625-5632, 1993.
372. Winkles JA, Gay CG. Regulated expression of PDGF A-chain mRNA in human saphenous vein smooth muscle cells. Biochem. Biophys. Res. Comm. 180; 519-524, 1991.

373. Colamonici OR, Trepel JB, Vidal CA, Neckers LM. Phorbol ester induces c-*sis* gene transcription in stem cell line K-562. Mol. Cell. Biol. 6: 1847-1850, 1986.
374. Alitalo R, Andersson LC, Betsholtz C, Nilsson K, Westermark B, Heldin C-H, Alitalo K. Induction of platelet-derived growth factor gene expression during megakaryoblastic and monocytic differentiation of human leukemia cell lines. EMBO J. 6: 1213-1218, 1987.
375. Sariban E, Kufe D. Expression of the platelet-derived growth factor 1 and 2 genes in huma myeloid cell lines and monocytes. Cancer Res. 48: 4498-4502, 1988.
376. Gay CG, Winkles JA. Heparin-binding growth factor-1 stimulation of human endothelial cells induces platelet-derived growth factor A-chain gene expression. J. Biol. Chem. 265: 3284-3292, 1990.
377. Kourembanas S, Faller DV. Platelet-derived growth factor production by human umbilical vein endothelial cells is regulated by basic fibroblast growth factor. J. Biol. Chem. 264: 4456-4459, 1989.
378. Schöllmann C, Grugel R, Tatje D, Hoppe J, Folkman J, Marmé D, Weich HA. Basic fibroblast growth factor modulates the mitogenic potency of the platelet-derived growth factor (PDGF) isoforms by specific upregulation of the PDGF α-receptor in vascular smooth muscle cells. J. Biol. Chem. 267: 18032-18039, 1992.
379. Harsh GR, Kavanaugh WM, Starksen NF, Williams LT. Cyclic AMP blocks expression of the c-*sis* gene in tumor cells. Oncogene Res. 4: 65-73, 1989.
380. Raines EW, Dower SK, Ross R. Interleukin-1 mitogenic activity for fibroblasts and smooth muscle cells is due to PDGF-AA. Science 243; 393-396, 1989.
381. Suzuki H, Shibano K, Okane M, Kono I, Matsui Y, Yamane K, Kashiwagi H. Interferon-γ modulates levels of c-*sis* (PDGF B-chain), PDGF A-chain, and IL-1ß genes in human vascular endothelial cells. Am. J. Pathol. 134: 35-43, 1989.
382. Kovacs EJ, Van Stedum S, Neuman JE. Selective induction of PDGF gene expression in peritoneal macrophages by interleukin-2. Immunobiology 190: 263-274, 1994.
383. Hajjar KA, Hajjar DP, Silverstein RL, Nachman RL. Tumor necrosis factor-mediated release of platelet-derived growth factor from cultured endothelial cells. J. Exp. Med. 166: 235-245, 1987.
384. Haynes AR, Shaw RJ. Dexamethasone-induced increase in platelet-derived growth factor (B) mRNA in human alveolar macrophages and myelomonocytic HL60 macrophage-like cells. Am. J. Resp. Cell Mol. Biol. 7: 198-206, 1992.
385. Haraguchi T, Alexander DB, King DS, Edwards CP, Firestone GL. Identification of the glucocorticoid suppressible mitogen from rat hepatoma cells as an angiogenic platelet-derived growth factor A-chain homodimer. J. Biol. Chem. 266: 18299-18307, 1991.
386. Keating MT, Williams LT. Autocrine stimulation of intracellular PDGF receptors in *v-sis* transformed cells. Science 239: 914-916, 1988.

2

OUTLINE OF THE THESIS

CHAPTER 2

OUTLINE OF THE THESIS

The aim of the studies described in this thesis was to get more insight into the regulation of platelet-derived growth factor (PDGF) chain and PDGF receptor expression in normal and malignant mesothelial cell lines. These cell lines constitute a good and relevant model system for such a study, because of their characteristic expression patterns and because of the important role that PDGF probably plays in the pathogenesis of mesothelioma.

In **Chapter** 3.1 the differential PDGF chain and receptor mRNA expression patterns of normal and malignant mesothelial cells are presented. The co-expression of PDGF B-chain and PDGF ß-receptor mRNA in the mesothelioma cell lines, but not in the untransformed mesothelial cells, is suggestive for a role for the PDGF B-chain as an autocrine growth factor in mesothelioma cells.

To study the expression of PDGF and PDGF receptor proteins, immunocytochemical stainings were also performed. The results of these stainings with antibodies directed against PDGF and the PDGF receptor subtypes in these cell lines are given in **Chapter** 3.2. Furthermore, to investigate whether the observed expression pattern in the cell lines reflects the actual expression of reactive and malignant mesothelial cells *in vivo*, effusions and frozen tissue sections are studied as well. Collectively, the expression data in these uncultured cells indicate that the mesothelial cell lines indeed form a good model system for studies of the role of PDGF in mesothelioma.

Transcriptional and post-transcriptional regulation of PDGF chain and receptor mRNA are important mechanisms in regulating the biological action of PDGF in (tumour) cells. **Chapter** 4.1 describes the regulation of the different PDGF A-chain mRNA levels in normal and malignant mesothelial cells and the alternative splicing of PDGF A-chain transcripts in these cell lines. In **Chapter** 4.2 the PDGF B-chain promoter region is studied in detail by *in vivo* footprint and reporter gene analysis. Furthermore, several DNaseI hypersensitive sites are mapped in regions upstream and downstream of the transcription unit in both normal and malignant mesothelial cells. The contribution of these hypersensitive sequences as potential regulatory elements in PDGF B-chain mRNA expression in mesothelioma cells is evaluated in **Chapter** 4.2 as well. Finally, in **Chapter** 4.3 regulation of the differential PDGF receptor messenger expression between normal and malignant mesothelial cell lines is described.

It is generally accepted that in tumorigenesis both oncogenes and tumour suppressor genes are involved. The WT1 (Wilms tumour) gene is one of these tumour suppressor genes. The product encoded by this gene displays DNA binding capacity. It seems to function in repression of transcription of target genes that contain a consensus DNA binding sequence. Expression of the WT1 gene is seen in a limited set

of tissues during embryonal development, e.g. the kidneys, the gonads, and the mesothelium. In **Chapter** 5.2 WT1 mRNA expression is analyzed in a panel of normal mesothelial cell lines. To see whether WT1 may play a role in mesothelial carcinogenesis, WT1 expression as well as the possible occurrence of point mutations, deletions, and gene rearrangements in WT1 are also studied in malignant mesothelioma cell lines (**Chapter** 5.2). Furthermore, as IGF2 and also PDGF A-chain have been mentioned as target genes for regulation by WT1, the possible relationship between the expression levels of these genes in the mesothelial cell lines are evaluated.

In the general discussion (**Chapter** 6) the results of the previous chapters are discussed with regard to data presented in the literature. Special attention is paid to the usefulness of mesothelioma cell lines as a model system to study regulation of PDGF chains and receptors. Furthermore, the contribution of factors like PDGF and WT1 to the pathogenesis of mesothelioma is evaluated in the context of other factors that may play a role in this malignancy.

3

EXPRESSION OF PDGF CHAINS AND RECEPTORS IN MESOTHELIAL CELLS *IN VITRO* AND *IN VIVO*

CHAPTER 3.1

Expression of PDGF chains and PDGF receptors in human malignant and normal mesothelial cell lines

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Expression of PDGF chains and PDGF receptors in human malignant and normal mesothelial cell lines. M.A. Versnel, A.W. Langerak, Th.H. van der Kwast, H.C. Hoogsteden, A. Hagemeijer. ©ERS Journals Ltd 1993.

ABSTRACT: A panel of human malignant mesothelioma cell lines and normal mesothelial cells was investigated for the expression of the genes coding for the platelet-derived growth factor (PDGF) A-chain, PDGF B-chain, PDGF α-receptor and PDGF β-receptor. The human malignant mesothelioma cell lines were found to express the PDGF A-chain, PDGF B-chain and PDGF β-receptor. Normal mesothelial cell lines were found to express the PDGF A-chain at a low level and the PDGF α-receptor. The PDGF β-receptor expression in normal mesothelial cells is generally weak to undetectable and sometimes stronger. The coexpression of PDGF B-chains and PDGF β-receptors on human malignant mesothelioma cell lines and the absence of PDGF B-chain expression in normal mesothelial cells suggests that the homo dimeric PDGF-BB protein can function as an autocrine growth factor and play a role in the stimulation of the growth of the tumour cells. In cultured normal mesothelial cells, on the other hand, PDGF-AA acting *via* the PDGF α-receptor may be involved in autocrine growth stimulation.

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normal mesothelial cell lines
platelet-derived growth factor chains
PDGF receptors

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Human malignant mesotheliomas are mesodermally derived tumours, most frequently encountered in the pleura and thought to develop from mesothelial cells. A panel of human malignant mesothelioma cell lines was established from pleural effusions and tumour tissue of malignant mesothelioma patients [1]. Normal mesothelial cells were cultured from pleural effusions of noncancerous patients or sheets of mesothelial tissue from thoracic surgery [1]. These normal and malignant mesothelial cell lines were used as a model for the study of the malignant transformation of mesothelial cells with emphasis on the expression of platelet-derived growth factor (PDGF) and PDGF receptors.

PDGF is a growth factor for mesenchymal cells and expression of PDGF has been found in a variety of normal and malignant cells [2, 3]. Two PDGF chain genes have been identified: the PDGF A-chain gene [4] and the PDGF B-chain gene [5, 6]. The PDGF B-chain is almost identical to the c-*sis* oncogene and is 60% similar to the PDGF A-chain gene [4]. All possible dimeric combinations of A- and B-chains have been identified [2, 3]. Binding experiments with different isoforms of PDGF revealed the existence of two distinct receptor types, denoted PDGF α- and β-receptor [7, 8]. The PDGF α-receptor binds all dimeric combinations (AA, AB and BB), whilst the PDGF β-receptor binds PDGF B-chains and no A-chains [9, 10]. Dimerization of PDGF receptors can result in three different PDGF receptor dimers (αα, αβ and ββ receptors) [10–12].

We investigated our panel of human normal and malignant mesothelial cell lines for the expression of PDGF chains and PDGF receptor genes.

Materials and methods

Cell lines

A panel of human malignant mesothelioma cell lines and normal mesothelial cell lines has been established, characterized and cultured as described previously [1, 13]. All malignant mesothelioma cell lines had a highly abnormal karyotype, whereas the normal mesothelial cell cultures had normal karyotypes [1].

Northern blot analysis and probes

Ribonucleic acid (RNA) isolation, Northern blot analysis and the probes used were described previously [13, 14].

Immunoprecipitation and radioreceptor analysis

Immunoprecipitation and radioreceptor analysis with ^{125}I-PDGF were performed as described previously [14].

Results

Expression of PDGF receptors and PDGF chains in malignant mesothelioma cell lines

RNA was isolated from a panel of human malignant mesothelioma cell lines. A Northern blot with total RNA from five of our malignant mesothelioma cell lines (Mero-14, Mero-25, Mero-41, Mero-72 and Mero-82), the normal mesothelial cell line NM-1 and placental RNA was hybridized to probes for PDGF α-receptor, PDGF β-receptor, PDGF B-chain and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (fig. 1). The PDGF α-receptor probe hybridized to a band of 7 kb in NM-1 and after long exposure to placental RNA. The malignant mesothelioma cell lines did not have a detectable level of PDGF α-receptor expression. Hybridization with probes for the PDGF β-receptor and PDGF B-chain at the same time revealed a variable level of expression of the PDGF β-receptor and PDGF B-chain in all malignant mesothelioma cell lines and human placenta. NM-1 did not show a detectable PDGF β-receptor or PDGF B-chain expression. The PDGF A-chain gene was expressed in all malignant mesothelioma cell lines (data not shown).

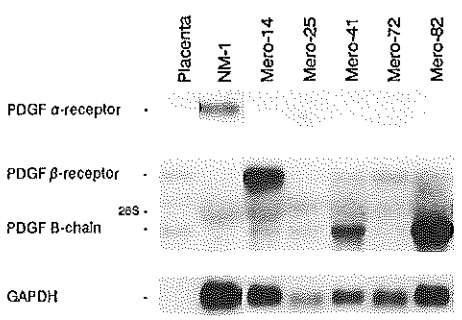

Fig. 1. – Northern blot analysis of 25 μg total RNA from placenta, a normal mesothelial cell line (NM-1) and five malignant mesothelioma cell lines subsequently hybridized to a ^{32}P-labelled PDGF α-receptor probe, PDGF β-receptor probe and at the same time a PDGF B-chain probe and a GAPDH probe. RNA: ribonucleic acid; PDGF: platelet-derived growth factor; GADPH: glyceraldehyde-3-phosphate dehydrogenase.

Expression of PDGF receptors and PDGF chains in normal mesothelial cell lines

Figure 2 shows RNA isolated from five different normal mesothelial cell lines NM-9, NM-10, NM-11, NM-12, NM-13 and the malignant mesothelioma cell line mero-82 hybridized with probes for the genes encoding the PDGF B-chain, PDGF α-receptor, PDGF β-receptor and GAPDH. In contrast to mero-82, none of the normal mesothelial cell lines showed a specific band after hybridization with the PDGF B-chain probe. The PDGF α-receptor messenger RNA (mRNA) was clearly detectable in all five normal mesothelial cell lines, whilst this band was absent in mero-82. The PDGF β-receptor was expressed in the malignant mesothelioma cell line and in one of the five (NM-11) normal mesothelial cell lines. The other four normal mesothelial cell lines (NM-9, -10, -12 and -13) did not show a significant signal. The PDGF A-chain gene was expressed in all five normal mesothelial cell lines at a weaker level than generally observed in malignant mesothelioma cell lines (data not shown).

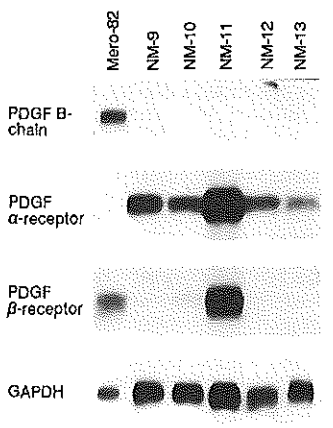

Fig. 2. – Northern blot analysis of 25 μg total RNA from a malignant mesothelioma cell line (Mero-82) and five normal mesothelial cell lines subsequently hybridized to a ^{32}P-labelled PDGF B-chain probe, PDGF α-receptor probe, PDGF β-receptor probe and a GAPDH probe. For abbreviations see legend to figure 1.

Expression of PDGF receptor proteins

The presence of PDGF α- and PDGF β-receptors in normal and malignant mesothelial cell lines was also demonstrated by immunoprecipitation and radioreceptor analysis with ^{125}I-labelled PDGF. Immunoprecipitation of metabolically labelled cells revealed expression of PDGF β-receptors in the malignant mesothelioma cell lines and predominantly PDGF α-receptors in the normal mesothelial cell lines (data not shown). Radioreceptor analysis showed the presence of the expressed receptors on the membrane of the cells (data not shown).

Discussion

Expression of the PDGF A-chain, PDGF B-chain and PDGF β-receptor genes was demonstrated in a panel of human malignant mesothelioma cell lines. The PDGF α-receptor was undetectable in these cell lines. In contrast, normal mesothelial cell lines expressed predominantly the PDGF α-receptor, the PDGF A-chain gene and no PDGF B-chain gene.

All twelve human malignant mesothelioma cell lines established and investigated in our department for

PDGF receptor and PDGF chain expression were found to express the genes for the PDGF B-chain, PDGF A-chain and the PDGF β-receptor [13, 14]. So far we have studied ten different normal mesothelial cell lines and found no expression of the PDGF B-chain gene, a weak PDGF A-chain expression and a strong PDGF α-receptor expression ([13, 14] and this paper). Two of these ten normal mesothelial cell lines were found to clearly express the PDGF β-receptor gene, whilst the others had a weak to undetectable level of this messenger RNA (mRNA). GERWIN *et al.* [15], also found expression of the PDGF B-chain gene in six malignant mesothelioma cell lines. However, no data on the PDGF receptor expression in these cell lines are yet available. A panel of five human malignant mesothelioma cell lines isolated in Western Australia was found to have a similar expression pattern of PDGF chains and PDGF receptors as our cell lines (Garlepp, unpublished results). Only one of these cell lines did not have PDGF B-chain mRNA. Thus, it seems that coexpression of the genes for PDGF A-chain, PDGF B-chain and the PDGF β-receptor is a general property of human malignant mesothelioma cell lines, with few exceptions. However, as a malignant mesothelioma is strongly related to asbestos exposure and these cell lines were isolated from patients with a different history of asbestos exposure (duration, load and kind of asbestos) exceptions on the observed pattern of expression could be due to variation in exposure. Furthermore, it is our experience that the diagnosis of malignant mesothelioma is still difficult and should be confirmed by different pathologists in order to avoid the use of erroneously named malignant mesothelioma cell lines.

The coexpression of PDGF B-chains and PDGF β-receptors on human malignant mesothelioma cell lines and the absence of PDGF B-chain expression in normal mesothelial cell lines suggests that PDGF-BB can function as an autocrine growth factor and play a role in the stimulation of the growth of the tumour cells. However, in cultured normal mesothelial cells it is possible that PDGF-AA acting *via* the PDGF α-receptor is involved in autocrine growth stimulation. Future investigations will be directed towards the interference in the autocrine growth stimulation and study of the expression of PDGF chains and PDGF receptors in fresh tumour material.

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References

1. Versnel MA, Bouts MJ, Hoogsteden HC, van der Kwast ThH, Delahaye M, Hagemeijer A. – Establishment of human malignant mesothelioma cell lines. *Int J Cancer* 1989; 44: 256–260.

2. Heldin C-H, Westermark B. – Platelet-derived growth factor: mechanism of action and possible *in vivo* function. *Cell Regul* 1990; 1: 555–566.

3. Raines WE, Bowen-Pope DF, Ross R. *In*: Sporn M.B., Roberts A.B., eds. Peptide growth factors and their receptors. Handbook in experimental pharmacology. Vol 95, part I. Heidelberg, Springer Verlag 1990; pp. 173–262.

4. Betsholtz C, Johnsson A, Heldin C-H, Westermark B, Lind P, Urdea MS, Eddy R, Shows TB, Philpott K, Mellor AL, Knott TJ, Scott J. – cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumour cell lines. *Nature* 1986; 320: 695–699.

5. Doolittle RF, Hunkapiller MW, Hood LE, Devare SG, Robbins KC, Aaronson SA, Antoniades HN. – Simian sarcoma virus one gene, *v-sis*, is derived from the gene (or genes) encoding a platelet-derived growth factor. *Science* 1983; 221: 275–277.

6. Waterfield MD, Scrace GT, Whittle N, Stroobant P, Johnsson A, Wasteson A, Westermark B, Heldin C-H, Huang JS, Deuel TF. – Platelet-derived growth factor is structurally related to the putative transforming protein p28^{sis} of simian sarcoma virus. *Nature* 1983; 304: 35–39.

7. Hart CE, Forstrom JW, Kelly JD, Seifert RA, Smith HA, Ross R, Murray MJ, Bowen-Pope DF. – Two classes of PDGF receptor recognize different isoforms of PDGF. *Science* 1988; 240: 1529–1531.

8. Heldin C-H, Bäckström G, Östman A, Hammacher A, Rönnstrand L, Rubin K, Nistér M, Westermark B. – Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types. *EMBO J* 1988; 7: 1387–1393.

9. Hammacher A, Nistér M, Westermark B, Heldin C-H. – A human glioma cell line secretes three structurally and functionally different dimeric forms of platelet-derived growth factor. *Eur J Biochem* 1988; 176: 179–186.

10. Seifert RA, Hart CE, Phillips PE, Forstrom JW, Ross R, Murray MJ, Bowen-Pope D. – Two different subunits associate to create isoform-specific platelet-derived growth factor receptors. *J Biol Chem* 1989; 264: 8771–8778.

11. Heldin C-H, Ernlund A, Rorsman C, Rönnstrand L. – Dimerization of B type PDGF receptors occurs after ligand binding and is closely associated with receptor kinase activation. *J Biol Chem* 1989; 264: 8905–8912.

12. Bishayee S, Majumdar S, Khire J, Das M. – Ligand-induced dimerization of the platelet-derived growth factor receptor. Monomer-dimer interconversion occurs independent of receptor phosphorylation. *J Biol Chem* 1989; 264: 11699–11705.

13. Versnel MA, Hagemeijer A, Bouts MJ, van der Kwast ThH, Hoogsteden HC. – Expression of c-*sis* (PDGF B-chain) and PDGF A-chain genes in ten human malignant mesothelioma cell lines derived from primary and metastatic tumors. *Oncogene* 1988; 2: 601–605.

14. Versnel MA, Claesson-Welsh L, Hammacher A, Bouts MJ, van der Kwast ThH, Eriksson A, Willemsen R, Weima SM, Hoogsteden HC, Hagemeijer A, Heldin C-H. – Human malignant mesothelioma cell lines express PDGF β-receptors whereas cultured normal mesothelial cells express predominantly PDGF α-receptors. *Oncogene* 1991; 6: 2005–2011.

15. Gerwin BI, Lechner JF, Reddel RR, Roberts AB, Robbins KC, Gabrielson EW, Harris CC. – Comparison of production of transforming growth factor-β and platelet-derived growth factor by normal human mesothelial cells and mesothelioma cell lines. *Cancer Res* 1987; 47: 6180–6184.

CHAPTER 3.2

EXPRESSION OF PDGF AND PDGF RECEPTORS IN HUMAN MALIGNANT MESOTHELIOMA *IN VITRO* AND *IN VIVO*[*]

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ABSTRACT

The expression of platelet-derived growth factor (PDGF) and PDGF receptors was studied in human normal and malignant mesothelial cells *in vitro* and *in vivo*. Staining with anti-cytokeratin and ME1 antibodies and ultrastructural analysis confirmed the mesothelial nature of the cell lines used to study PDGF and PDGF receptor expression *in vitro*. Using antibodies, mesothelioma cell lines were found to express PDGF and both the PDGF α- and ß-receptor, whereas cultured normal mesothelial cells expressed PDGF and PDGF α-receptor. This PDGF and PDGF receptor staining pattern largely reflects the earlier described mRNA expression in these cell lines. The only exception was the immunocytochemical detection of PDGF α-receptors in the mesothelioma cell lines, which is different from the inability to detect α-receptor transcripts on Northern blots.

Expression was also investigated in mesothelial cells *in vivo*. Expression of PDGF was observed in malignant mesothelioma cells on frozen tissue sections. In pleural effusions a double immunofluorescence staining procedure for PDGF and epithelial membrane antigen (EMA), revealed PDGF expression by EMA-positive malignant mesothelioma cells. PDGF β-receptors and occasionally PDGF α-receptors were detected in frozen tissue sections of malignant mesotheliomas, whereas mesothelioma cells in effusions showed faint expression of only the PDGF ß-receptor. In contrast, in effusions containing non-malignant mesothelial cells only a very low level of PDGF α-receptor could be detected.

Taken together these results indicate that the pattern of PDGF and PDGF receptor expression in mesothelial cells *in vivo* largely corresponds with expression of PDGF and its receptors *in vitro*. Malignant mesothelioma cell lines thus constitute a good model system for studies on the role of PDGF in this malignancy. Furthermore, the data in the present paper are consistent with the idea that an autocrine growth stimulatory effect of PDGF via PDGF receptors may play a role in the pathogenesis of malignant mesothelioma.

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Keywords: malignant mesothelioma; platelet-derived growth factor; platelet-derived growth factor receptor; immunostaining.

INTRODUCTION

Human malignant mesothelioma is a tumour of mesothelial origin, which is seen most frequently in the lining of the coelomic cavities. Epidemiological studies have indicated that the incidence of malignant mesothelioma is strongly related to exposure to asbestos fibers (1), but the mechanism by which cells become transformed by asbestos is largely unknown. Although in recent years asbestos usage has been greatly diminished, the incidence of malignant mesothelioma is still increasing. This is due to the long latency period (15-45 years) between asbestos exposure and tumour development.

Platelet-derived growth factor (PDGF) is mitogenic for cells of mesenchymal origin (2,3). The dimeric PDGF molecule consists of two disulfide bounded polypeptides, which are encoded by two distinct genes, the PDGF A- and B-chain genes. All three possible dimeric isoforms of PDGF (AA, AB and BB) were found to be produced by various normal cell types (4-6). Two distinct receptors for PDGF were identified: the PDGF α-receptor which binds all isoforms and the PDGF β-receptor which binds just PDGF-BB (7,8). *In vivo* PDGF seems to play a role in wound healing and several non-malignant and malignant pathological disorders (reviewed by Heldin, 9). Expression of PDGF and its receptors in tumour cells has been considered suggestive for an autocrine growth stimulation. Recently, evidence was presented for autocrine stimulation by PDGF, as dominant-negative mutants of the PDGF ligand were shown to revert the transformed phenotype of two astrocytoma cell lines (10). Similarly, an autocrine loop in a glioblastoma cell line was shown to be blocked by PDGF neutralizing antibodies (11).

In previous studies, we suggested that an autocrine PDGF-dependent loop may play a role in the pathogenesis of malignant mesothelioma. This was based on results from studies on mRNA expression of PDGF chains and receptors in normal and malignant mesothelial cell lines. Human malignant mesothelioma cell lines were found to express PDGF A-chain and B-chain genes, whereas in contrast no PDGF B-chain mRNA and a low level of PDGF A-chain mRNA expression were found in cultured normal mesothelial cells (12,13). Furthermore, expression of the PDGF β-receptor was observed in human malignant mesothelioma cell lines, while normal mesothelial cells were found to predominantly express the PDGF α-receptor (14). In this paper we report on the immunocytochemical and immunohistochemical analysis of mesothelial cell lines, pleural effusion cells from mesothelioma patients as well as patients without a malignancy, and frozen tissue sections of malignant mesothelioma patients, with antibodies directed against PDGF and PDGF receptors. The resulting staining pattern is discussed in comparison with the previously reported mRNA expression pattern.

MATERIAL AND METHODS

Cell lines and culture conditions

Normal (NM) and malignant (Mero) human mesothelial cell lines were described previously (13,15,16).

Culture conditions of the cell lines were described as well. Fibroblasts, derived from skin connective tissue, were used for comparison with cultured normal mesothelial cells. Cytogenetic analysis on the normal mesothelial cell lines revealed no clonal chromosomal aberrations, whereas analysis of the malignant cell lines demonstrated many abnormalities (13).

Patient material and diagnosis

Pleural effusion cells were collected and harvested as described (15). Autopsy was performed within two hours after death. Immediately after autopsy, tumour material was frozen in liquid nitrogen and stored at -70°C. Diagnosis was established by routine cytology and always confirmed ultrastructurally and/or histologically.

Immunofluorescence staining

For immunofluorescence (IF) staining pleural effusion cells were used either immediately or after storage in RPMI medium with 40% FCS and 10% dimethyl sulfoxide at -196°C. At a later stage after thawing, these cells were washed twice in phosphate buffered saline (PBS) with 0.5% bovine serum albumin (BSA). Cytospin preparations were made from 1-2 x 10^6 cultured or uncultured cells/ml phosphate-buffered saline (PBS) with 0.5 % bovine serum albumin (BSA) and 5 % human serum albumin (HSA). The slides were fixed in acetone for 10 min at room temperature (RT) and airdried. For a single IF staining the slides were incubated with the first step antibody for 30 min in a moist chamber at RT and subsequently washed in PBS for 15 min at RT. After incubation with second step fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse (GαM-FITC) or goat-anti-rabbit (GαR-FITC) antiserum with 10% pooled human serum for 30 min in a moist chamber at RT, the slides were washed again and were mounted in glycerol/PBS (9:1) with 1 mg/ml phenylenediamine (BDH Chemicals, Poole, UK) and sealed under coverslips with paraffinwax. Incubations with just second step GαM-FITC and GαR-FITC antibodies or with non-specific first step antibodies were included for control. At least 150 cells were analyzed by fluorescence microscopy and the percentage of cells staining above background levels was determined. For the double IF staining (PDGF and EMA) slides were incubated (30 min, RT) with the antibody PGF007 (Mochida Pharmac. Co. Ltd., Tokyo, Japan) and washed in PBS. Then the slides were incubated for 30 min at RT with a FITC-conjugated goat-anti-mouse IgG1 antibody (SBA, Birmingham, AL). Subsequently the slides were washed and incubated (30 min, RT) with an anti-EMA antibody (Dako, Glostrup, Denmark). After washing the slides were finally incubated for 30 min at RT with an tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat-anti-mouse IgG2a antibody (SBA). Incubations with non-specific first step antibodies were included for control.

Immunohistochemistry

Immunohistochemical staining was largely performed as described by Versnel et al. (17). The only exception is that subsequent to incubation with diamonobenzidine for visualization of enzyme activity, an enhancement was performed with 0.5% $CuSO_4.5H_2O$ in 0.9% NaCl for 5 min.

Antibodies

In this study mouse monoclonal antibodies RGE53 (18) (undiluted supernatant) directed against cytokeratin 18, ME1(19) (undiluted supernatant) against a mesothelial membrane antigen, and anti-EMA (1:80; Dako, Glostrup, Denmark) against epithelial membrane antigen, were used. Mouse monoclonal antibody PGF007 (20) (1:400) and rabbit polyclonal antibody Zp215 (1:50; Genzyme, Cambridge, MA) were used to detect PDGF. Although reported to be specific for PDGF-BB and PDGF-AB it is our experience that the reactivity of both these antibodies could be blocked by preincubation with native PDGF-BB as well as PDGF-AA (our own observations). Moreover, PGF007 has also been described to recognize both PDGF-BB and PDGF-AA in A-chain and B-chain transfected cell lines (21). Rabbit polyclonal Zp214 (1:100; Genzyme), directed against PDGF-AA, could be blocked only very weakly by preincubation with PDGF-BB. For detection of PDGF α-receptor and PDGF ß-receptor proteins, respectively, mouse monoclonals 1264-00 (clone PR292; 1:60; Genzyme) and 1263-00 (clone PR7212; 1:50; Genzyme) were used. These were described as PDGF-receptor type-specific antibodies (22).

RESULTS

Characterization of mesothelial cell lines

Our panel of normal (NM) and malignant (Mero) mesothelial cell lines was characterized by various methods to obtain evidence for their mesothelial origin. Human fibroblasts were used as controls. All mesothelial cell lines were positive for the anti-cytokeratin 18 antibody RGE53 (Figure 1a, and Table 1). Antibody ME1, which has been reported to react with a mesothelial specific membrane antigen, stained all normal mesothelial cell lines and the majority of malignant mesothelioma cell lines (Figure 1c,d and Table 1). Anti-EMA positive cells were observed in 50 % of the malignant mesothelioma cell lines, but no positive cells could be found in the normal mesothelial cell lines (Table 1). Fibroblasts did not stain with RGE53, ME1 or anti-EMA. TEM examination of several mesothelial cell lines revealed the presence of several features (microvilli with a more or less slender phenotype, glycogen granules, intermediate filaments and occasionally junctional complexes) that are consistent with a mesothelial origin (data not shown). When EGF and HC were left out of the standard culture medium for three days, most of the normal mesothelial cell lines switched their morphology from fibrous to epithelial (Table 1), similarly to the original description of Connell and Rheinwald (23). Fibroblasts did not demonstrate this morphological switch.

Expression of PDGF and PDGF receptors in mesothelial cell lines

In the various mesothelial cell lines expression of PDGF and both PDGF α- and ß-receptors was analyzed by immunofluorescence staining (Table 1). In all cases clear staining (85-100% of cells) was seen with the anti-PDGF antibody PGF007. Reactivity with the anti-PDGF-AA antibody Zp214 was observed in a high number of cells (80-100%) of normal and malignant mesothelial cell lines as well. When using an anti-PDGF α-receptor monoclonal antibody (1264-00) strong reactivity was observed in the normal mesothelial cell lines and in nearly all malignant mesothelioma cell lines (Figure 1e,f and Table 1). With anti-PDGF ß-receptor antibody 1263-00 the normal mesothelial cell lines demonstrated almost no reactivity (Figure 1g), whereas in the malignant mesothelioma lines the PDGF ß-receptor staining pattern was variable. In seven out of fourteen cell lines 100% positivity was observed (Figure 1h), whereas in the remaining seven no clear expression could be detected. Cultured fibroblasts reacted strongly with the PGF007, Zp214, and anti-PDGF α- and ß-receptor antibodies.

Expression of PDGF and PDGF receptors in pleural effusion cells

In an attempt to study expression of PDGF in mesothelioma cells *in vivo*, total RNA from pleural effusion cells of eight malignant mesothelioma patients was analyzed for the expression of PDGF B-chain mRNA by Northern blot analysis. In all cases the 3.5 kb PDGF B-chain transcript could be detected, but the level of expression was variable and did not correlate with the cytologically detected percentage of tumour cells. Moreover, expression could not be attributed exclusively to mesothelioma cells as other cell types like macrophages are also present in these effusions. In order to investigate expression of PDGF and its receptors at a single cell level, cytospin preparations of pleural effusion cells were

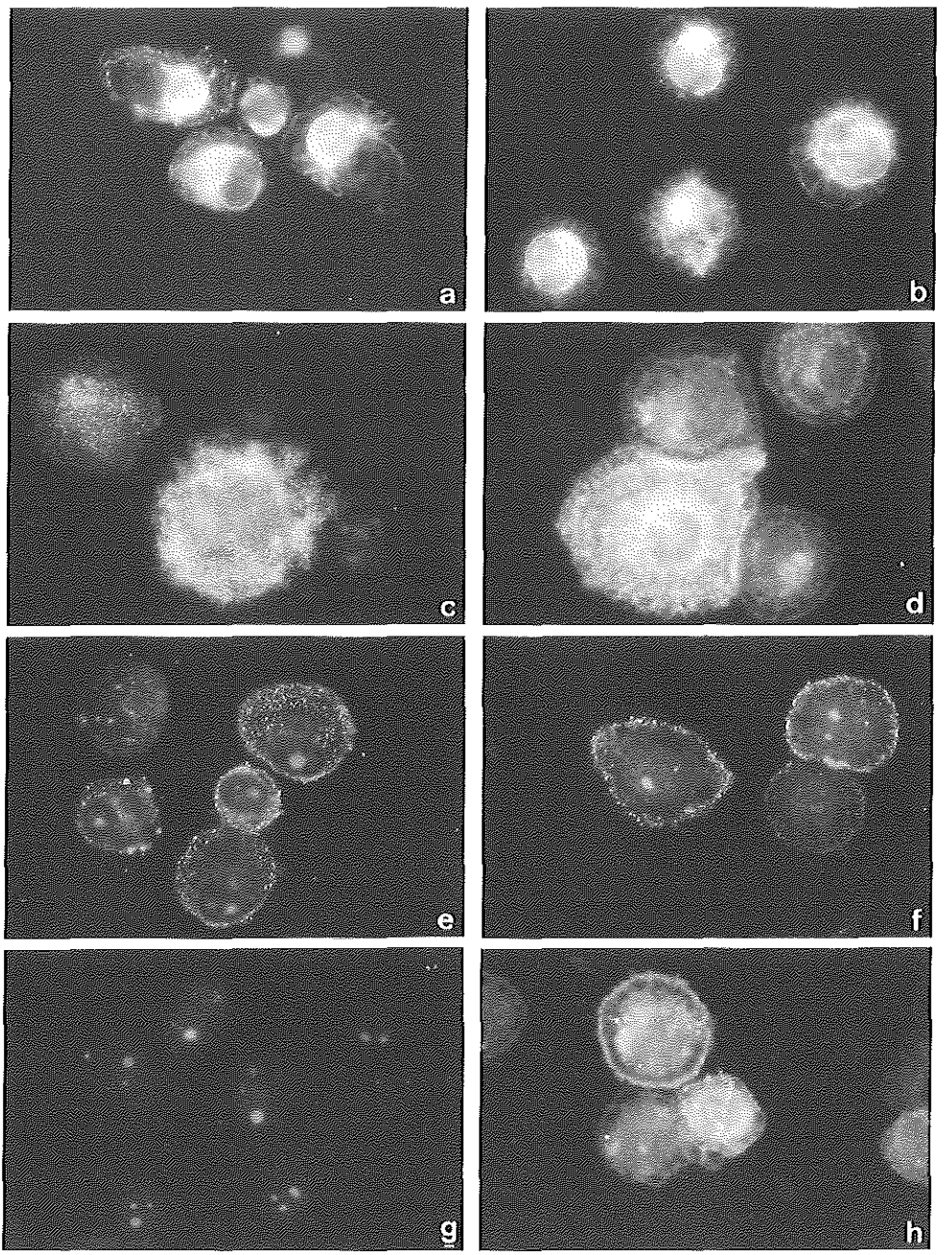

Figure 1. Immunofluorescence staining of normal human mesothelial cell line NM-21 (a,c,e,g) and human malignant mesothelioma cell line Mero-72 (b,d,f,h). (a,b) anti-cytokeratin 18 staining; (c,d) ME1 staining; (e,f) anti-PDGF α-receptor staining; (g,h) anti-PDGF ß-receptor staining. Magnification 500x.

TABLE 1. Immunofluorescence staining (% of cells staining positive) and morphological data of normal (NM) and malignant (Mero) mesothelial cell lines and cultured human fibroblasts.

cell line	source of material	subtype tumour	switch	RGE53 (CK18)[a]	ME1 (unknown)	anti-EMA (EMA)	PGF007 (PDGF)	Zp214 (PDGF)	1264-00 (PDGFαR)	1263-00 PDGFβR)
Mero-14	effusion	epithelial	NA	100	100	0	100	97	100	100
Mero-25	autopsy	epithelial	NA	100	100	4	100	100	66	0
Mero-41	effusion	epith.comp.	NA	100	100	69	100	100	91	100
Mero-48a	autopsy	biphasic	NA	100	100	0	100	100	54	0
Mero-48b	autopsy	biphasic	NA	100	100	100	100	100	100	93
Mero-48c	autopsy	biphasic	NA	100	100	77	100	100	100	100
Mero-72	biopsy	biphasic	NA	100	100	100	100	100	100	100
Mero-82	effusion	fibrous	NA	100	4	95	100	100	93	4
Mero-83	effusion	biphasic	NA	100	100	3	100	100	68	3
Mero-84	effusion	epithelial	NA	100	100	92	100	100	0	0
Mero-95	effusion	epith.comp.	NA	100	91	87	100	100	69	1
Mero-96	biopsy	fibrous	NA	6	0	4	100	100	100	100
Mero-123	autopsy	fibrous	NA	100	0	0	100	100	100	100
Mero-134	effusion	epith.comp.	NA	100	0	0	100	87	91	0
NM-1	effusion	NA	yes	94	42	ND	95	99	94	0
NM-2	tissue	NA	yes	76	42	ND	80	91	83	0
NM-3	effusion	NA	+/-	100	100	ND	86	100	96	1
NM-4	effusion	NA	+/-	100	53	ND	96	100	86	1
NM-5	tissue	NA	no	69	56	ND	78	95	84	3
NM-8	tissue	NA	yes	100	43	ND	97	ND	100	1
NM-9	tissue	NA	yes	100	46	0	100	100	100	0
NM-12	tissue	NA	yes	97	56	0	100	100	98	1
NM-21	ascites	NA	yes	100	43	0	100	100	80	0
human fibroblasts	connective tissue	NA	no	0	0	0	100	100	100	100

ND: not determined, NA: not applicable.
a. antigen recognized in parenthesis (see also Materials and Methods).

TABLE 2. Immunofluorescence staining of pleural effusions containing malignant mesothelioma cells (Me) or non-malignant mesothelial cells (M).

patient	cells[a]	anti-EMA[b] (EMA)[d]	PGF007[b] (PDGF)	anti-EMA/PGF007[c]	Zp215 (PDGF)	Zp214 (PDGF)	1264-00 (PDGFαR)	1263-00 (PDGFβR)
Me-7	1 – 25	+	+	+	+	+	-	+
Me-67	75 – 100	+	+	+	+	+	-	-
Me-69	50 – 75	+	+	+	+	+	-	+
Me-83	1 – 25	+	+	+	+	+	-	-
Me-84	75 – 100	+	+	+	+	+	-	+
Me-85	50 – 75	+	+	+	+	+	-	-
Me-103	75 – 100	+	+	+	+	+	-	+
Me-135	25 – 50	+	+	+	+	+	-	+
Me-160	50 – 75	+	+	+	+	+	-	+
Me-164	75 – 100	+	+	+	+	+	-	+/-
Me-169	75 – 100	+	+	+	+	+	ND	-
Me-178	50 – 75	+	+	+	+	+	-	+
Me-184	50 - 75	+	+	+	+	+	-	+
Me-186	1 – 25	+	+	+	+	+	-	-
Me-197[e]	1 – 25	+	+	+	+	+	-	-
M-12	1 - 25	-	+	-	+/-	+	+	+
M-17	1 - 25	+	+	ND	+	+	+	-
M-55	1 - 25	-	+	-	+	+	+	-
M-81	1 - 25	+	+	ND	+	+	+	-
M-82	1 - 25	-	+	-	+	+	+	-
M-83	50 - 75	+	+	+	+	+	+	-

+ staining; +/– doubtful staining; – no staining; ND: not determined.
a. cytologically detected percentage mesothelioma cells (Me) or non-malignant mesothelial cells (M).
b. immunofluorescence staining.
c. double immunofluorescence staining.
d. antigen recognized in parenthesis (see also Materials and Methods).
e. ascites.

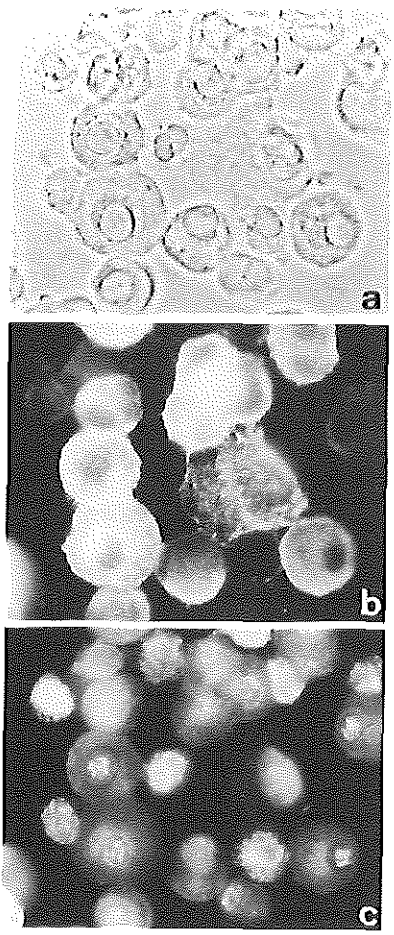

Figure 2. Double immunofluorescence staining for EMA and PDGF on pleural effusion cells of malignant mesothelioma patient Me-169. (a) phase contrast morphology; (b) EMA-positive cells (TRITC-labeled); (c) PDGF-positive cells (FITC-labeled). The three photographs represent the same microscopic field. Magnification 500x.

made and used for immunofluorescence staining. In Table 2 the staining results of pleural effusion cells of fifteen malignant mesothelioma patients are presented. The percentage of cytologically detected malignant mesothelioma cells in these effusions varies from less than 25% to nearly 100%. EMA expression was observed in all cases, but in several patients not all cytologically detected tumour cells were EMA^+. The staining pattern for EMA varied between dull cytoplasmic and bright membrane staining. In all cases the percentage $PDGF^+$ effusion cells, as detected with anti-PDGF antibody PGF007, was higher than the cytologically detected percentage malignant mesothelioma cells, due to cells with a macrophage-like appearance expressing PDGF as well. In order to prove that malignant mesothelioma cells in pleural effusions express PDGF, a double IF staining procedure for PDGF in combination with EMA was developed. With this double staining PDGF expression was seen in most EMA^+ cells from the various patients. In Figure 2 a double IF staining of pleural effusion cells from malignant mesothelioma patient Me-169 is presented. Most mesothelioma cells were found to be EMA^+ and $PDGF^+$ (Figure 2b,c), whereas cells demonstrating a macrophage-like morphology (Figure 2a) were EMA^- but $PDGF^+$ (Figure 2b,c). Stainings performed with the polyclonal anti-PDGF antibody Zp215 yielded staining results similar to the ones obtained with PGF007 (Table 2). Unfortunately, a double IF staining with EMA and Zp215 could not be performed as staining with the latter antibody was too faint. In all cases mesothelioma cells stained with the anti-PDGF-AA antibody Zp214. Mesothelial cells in effusions from patients without a malignancy reacted strongly with antibodies PGF007, Zp215 and Zp214 as well (Table 2). In non-malignant effusions staining of mesothelial cells for Zp214 was more intense than the staining for Zp215 and PGF007, whereas non-malignant mesothelial cells were only occasionally EMA-positive. Stainings with antibodies 1264-00 and 1263-00, directed against PDGF α- and ß-receptors, respectively, are also shown in Table 2. No PDGF α-receptors were observed in mesothelioma cells from pleural effusions, whereas PDGF ß-receptors could be detected in at least eight out of fifteen cases. However, the PDGF ß-receptor signal was often faint and not all tumour cells were positive. In non-malignant

effusions the opposite was observed, i.e. the mesothelial cells demonstrated no detectable PDGF ß-receptors and a faint PDGF α-receptor expression.

Expression of PDGF and PDGF receptors in frozen tissue sections

Frozen tissue sections of fifteen pleural malignant mesothelioma patients were stained using an immunoperoxidase technique with antibodies against EMA, PDGF and the PDGF receptors (Table 3). In Figure 3 stained tissue sections of patient Me-202 are presented. Thirteen mesothelioma tissues were EMA$^+$ (Figure 3a,b). PDGF was detected using antibodies PGF007 and Zp215. Using PGF007, PDGF$^+$ tumour cells were detected in twelve out of thirteen malignant mesotheliomas. The immunoreactivity with PGF007 was characterized by a perinuclear staining (Figure 3c,d). Except for a few cases, the results obtained with Zp215 were similar to those obtained with PGF007. In general, staining with PGF007 was more intense. No correlation could be observed between PDGF expression or EMA positivity and the tumour histology. The malignant mesothelioma tissue sections were stained with antibodies directed against the PDGF α- and β-receptors as well. In nine out of fourteen analyzed patients PDGF α-receptor expression was observed, although it should be noted that the staining intensity was variable (Figure 3e). In all cases the majority of mesothelioma cells were found to react with anti-PDGF β-receptor antibody 1263-00 (Figure 3f).

TABLE 3. Immunoperoxidase staining of frozen tissue sections from pleural malignant mesothelioma patients.

patient	histology	anti-EMA (EMA)[a]	PGF007 (PDGF)	Zp215 (PDGF)	1264-00 (PDGFαR)	1263-00 (PDGFβR)
Me-7	epithelial	+	-	+/-	+	+ +
Me-48	biphasic	+ +	+ + +	+ +	+/-	+
Me-66	biphasic	+ +	+ + +	+	+	+ +
Me-71	epithelial	+ + +	+ + +	+ + +	+	+ +
Me-72	biphasic	+ + +	ND	+ + +	+/-	+ +
Me-77	biphasic	+ +	ND	-	-	+/-
Me-84	epithelial	+	+ + +	+ + +	-	+
Me-97	biphasic	-	+	ND	ND	+
Me-122	biphasic	+/-	+ +	+	+	+
Me-123	fibrous	+	+ + +	+ + +	+/-	+
Me-128	biphasic	+	+ +	+/+ +	+/+ +	+ +
Me-137	biphasic	+/-	+ +	+/+ +	-	+
Me-200	biphasic	-	+ + +	+/-	-	+ +
Me-201	biphasic	+	+ +	-	-	+ +
Me-202	epithelial	+ +	+ + +	+/-	+/+ +	+ + +

+ + + very strong staining; + + strong staining; + moderate staining; +/- weak staining; - negative staining; ND: not determined.
a. antigen recognized in parenthesis (see also Materials and Methods).

DISCUSSION

Previously we reported on the mRNA expression pattern of PDGF chains and receptors in normal and malignant mesothelial cell lines (13,14). The mesothelial nature of these cell

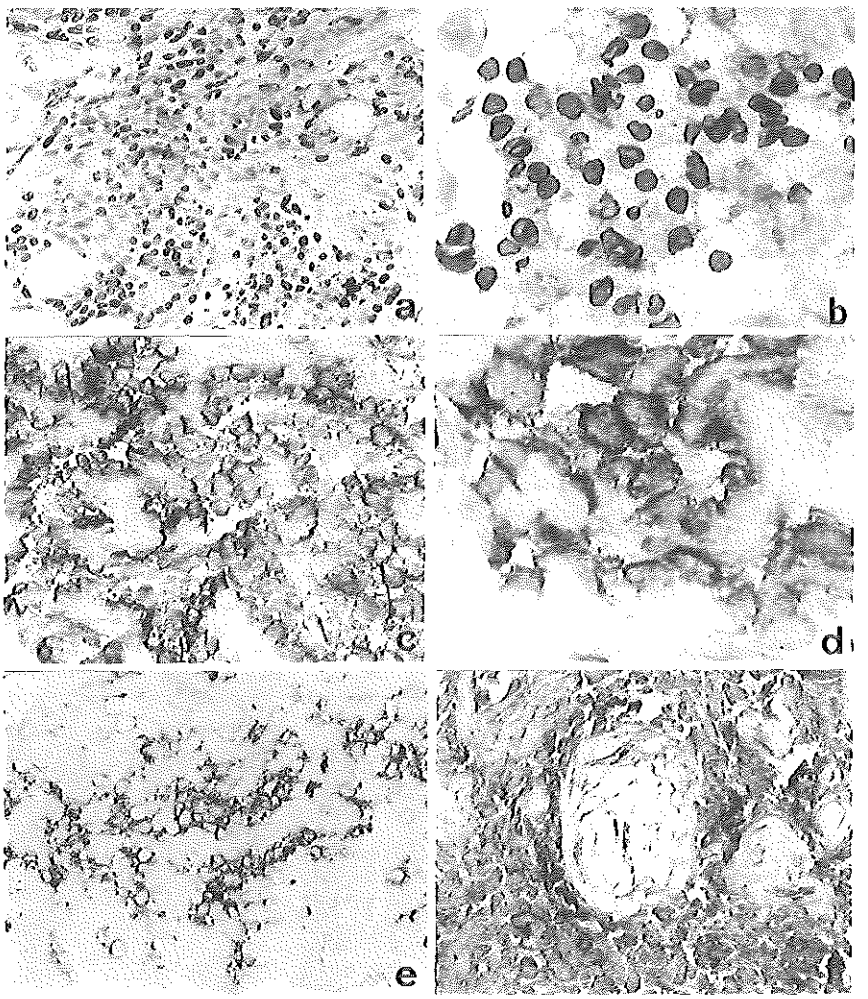

Figure 3. Immunohistochemical staining of frozen tissue sections of mesothelioma patient Me-202. (a,b) anti-PDGF (PGF007)-staining; (c,d) anti-EMA staining; (e) anti-PDGF α-receptor staining; (f) anti-PDGF ß-receptor staining. Magnification 250x (a,c,e,f) and 675x (b,d).

lines was confirmed in various ways. Staining with anti-cytokeratin and ME1 antibodies, as observed in cultured normal and malignant mesothelial cells (19,23-25), was seen in these mesothelial cell lines as well. In the present report the various cell lines were analyzed immunocytochemically with antibodies against the PDGF chains and receptors. The immunocytochemical detection of PDGF-AA (Zp214) and PDGF α-receptors (1264-00) and the lack of PDGF ß-receptors in normal mesothelial cell lines is consistent with the earlier observed mRNA pattern. Given the fact that PGF007 reactivity could also be blocked by native PDGF-AA (21 and our own observation), PGF007 most probably reacts with PDGF-

AA in normal mesothelial cells, since we could not detect PDGF B-chain transcripts in these cell lines using Northern blot and run off analysis (Langerak et al., in preparation). The staining pattern in the various mesothelioma cell lines largely confirmed the earlier reported expression of PDGF A-chain and PDGF B-chain mRNA in these cell lines. Furthermore, in those cell lines showing high levels of PDGF ß-receptor mRNA PDGF ß-receptors could clearly be detected immunocytochemically, whereas in cell lines in which lower or undetectable PDGF ß-receptor mRNA levels were observed PDGF ß-receptor proteins were almost or totally undetectable in the majority of cells. The immunocytochemical detection of PDGF α-receptors in nearly all mesothelioma cell lines in the present study is in contrast with their lack of PDGF α-receptor transcripts in Northern blot analysis (14). However, it does fit in with the detection of α-receptor mRNA in mesothelioma cell lines using the more sensitive nuclear run off (Langerak et al., in preparation) and RNase protection assays (26). Furthermore, together this also suggests that our earlier obtained data from radioreceptor assays should be interpreted as specific, although very low, binding of PDGF-AA to PDGF α-receptors in mesothelioma cell lines (14).

In order to test whether the cell lines constitute a relevant model system for the study of the role of PDGF and PDGF receptors in malignant mesothelioma *in vivo*, the expression pattern was analyzed in effusions and biopsies as well. Northern blot analysis of PDGF expression in total RNA of effusions from malignant mesothelioma patients appeared to be inconclusive due to the presence of contaminating cell types in these effusions, whereas RNA *in situ* hybridization on pleural effusion cells resulted in high background levels. Therefore, antibodies directed against PDGF and the PDGF receptors were used to analyze patient material. Given the specificities of the various anti-PDGF antibodies as described in Materials and Methods, we conclude that malignant mesothelioma cells in tissue sections express PDGF, which is at least partly of the PDGF-AA form. The same was shown for mesothelioma cells in pleural effusions using a double IF staining for PDGF and EMA. Mesothelial cells from non-malignant effusions were observed to express PDGF as well.

In addition to PDGF expression, we found membrane expression of PDGF β-receptors in tissue sections from all mesothelioma patients studied. Using the same antibody, Ramael et al. (27) found cytoplasmic and sometimes membrane expression of the ß-receptor in only half of the malignant mesotheliomas. This difference in reactivity and subcellular localization might result from the use of paraffin-embedded material by Ramael et al. (27). We also observed small groups of PDGF α-receptor$^+$ cells in nine out of fourteen cases analyzed, but due to the poor morphology in frozen tissue sections it remains unclear whether these are malignant mesothelioma cells or stromal cells. Thus far there have been no reports on PDGF α-receptor expression in malignant mesothelioma *in vivo*. In mesothelioma cells of pleural effusions we could not detect PDGF α-receptors, whereas expression of PDGF ß-receptors was seen in these cells in eight out of fifteen effusions. This may indicate that in some cases malignant mesothelioma cells have lost their PDGF β-receptor expression upon leaving the solid tumour tissue or that the expression in the exfoliated mesothelioma cells is below the detection limit. In non-malignant mesothelial cells in effusions only a faint PDGF α-receptor expression was seen. The fact that *in vitro* normal mesothelial cells clearly express PDGF α-receptors and mesothelioma cells both PDGF α- and ß-receptors, suggests that the levels of these receptors are upregulated in culture. Upregulation of the PDGF receptor in culture has been reported in fibroblasts and

smooth muscle cells (28). In general, the use of anti-PDGF and anti-PDGF receptor antibodies does not seem to be suitable for a reliable diagnosis of malignant mesothelioma on pleural effusions.

In this report we demonstrate that malignant mesothelioma cells, but not non-malignant mesothelial cells, co-express PDGF and PDGF β-receptors *in vitro* as well as *in vivo*. The *in vivo* data are thus consistent with the earlier suggestion that PDGF contributes to the pathogenesis of malignant mesothelioma in an autocrine way by activating PDGF β-receptors. In primary human lung carcinomas, but not in non-malignant epithelial cells, co-expression of PDGF B-chain and PDGF β-receptors was reported as well (29). Similarly, Hermanson et al. (30) demonstrated high expression of PDGF α-receptors and PDGF A- and PDGF B-chains in gliomas. PDGF expressed by the mesothelioma cells may exert a paracrine function as well. The possible expression of PDGF α-receptors on stromal cells on frozen tissue sections of mesotheliomas may be in support of this. Based on the strong PDGF ß-receptor expression in tumour-supporting stromal tissues, a paracrine function of PDGF in tumour growth has been suggested in gliomas (30,31) as well. Convincing evidence for a paracrine role of tumour-derived PDGF in stromal development came from a study in which nude mice were inoculated with PDGF B-chain-transfected human melanoma cells (32). These PDGF β-receptor$^-$, PDGF-BB$^+$ melanoma cells produced tumours with abundant supporting connective tissue without necrosis, whereas PDGF β-receptor$^-$, PDGF-BB$^-$ melanoma cells gave rise to tumours with necrotic areas and a poorly developed stroma.

In conclusion, this paper describes the immunofluorescence staining of PDGF and PDGF receptors in normal and malignant human mesothelial cell lines. The observed pattern largely reflects the earlier reported PDGF chain and receptor mRNA expression pattern in these cells. Furthermore, expression of PDGF and PDGF receptors in human malignant mesothelioma cells *in vivo* is similar to the expression observed *in vitro*. These data support the idea that an autocrine growth stimulatory effect of PDGF via PDGF receptors could play a role in the pathogenesis of malignant mesothelioma. Malignant mesothelioma cell lines thus constitute a good model system for future studies on the role of PDGF in this malignancy.

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REFERENCES

1. Wagner JC, Sleggs CA, Marchand P. Diffuse pleural mesothelioma and asbestos exposure in the North Western Cape Province. *Br J Ind Med* 1960; 17: 260-270
2. Ross R, Raines EW, Bowen-Pope DF: The biology of the platelet-derived growth factor. *Cell* 1986; 46: 155-169
3. Heldin C-H, Westermark B. Platelet-derived growth factor: mechanism of action and possible *in vivo* function. *Cell Regulation* 1990; 1: 555-566

4. Stroobant P, Waterfield MD. Purification and properties of porcine platelet-derived growth factor. *EMBO J* 1984; **12**: 2963-2967
5. Heldin C-H, Johnsson A, Wennergren S, Wernstedt C, Westermark B. A human osteosarcoma cell line secretes a growth factor structurally related to a homodimer of PDGF A-chains. *Nature* 1986; **319**: 511-514
6. Hammacher A, Hellman U, Johnsson A, Östman A, Gunnarsson K, Westermark B, Wästeson Å, Heldin C-H. A major part of platelet-derived growth factor purified from human platelets is a heterodimer of one A and one B chain. *J Biol Chem* 1988; **263**: 16493-16498
7. Hart CE, Forstrom JW, Kelly JD, Seifert RA, Smith RA, Ross R, Murray MJ, Bowen-Pope DF. Two classes of PDGF receptor recognize different isoforms of PDGF. *Science* 1988; **240**: 1529-1531
8. Heldin C-H, Bäckström G, Östman A, Hammacher A, Rönnstrand L, Rubin K, Nistér M, Westermark B. Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types. *EMBO J* 1988; **7**: 1387-1393
9. Heldin C-H. Structural and functional studies on platelet-derived growth factor. *EMBO J* 1992; **11**: 4251-4259
10. Shamah SM, Stiles CD, Guha A. Dominant-negative mutants of platelet-derived growth factor revert the transformed phenotype of human astrocytoma cells. *Mol Cell Biol* 1993; **13**: 7203-7212
11. Vassbotn F, Östman A, Langeland N, Holmsen H, Westermark B, Heldin C-H, Nistér M. Activated platelet-derived growth factor autocrine pathway drives the transformed phenotype of a human glioblastoma cell line. *J Cell Physiol* 1994; **158**: 381-389
12. Gerwin BI, Lechner JF, Reddel RR, Roberts AB, Robbins KC, Gabrielson EW, Harris CC. Comparison of production of transforming growth factor-beta and platelet-derived growth factor by normal human mesothelial cells and mesothelioma cell lines. *Cancer Res* 1987; **47**: 6180-6184
13. Versnel MA, Hagemeijer A, Bouts MJ, Van der Kwast ThH, Hoogsteden HC. Expression of c-sis (PDGF B-chain) and PDGF A-chain genes in ten human malignant mesothelioma cell lines derived from primary and metastatic tumors. *Oncogene* 1988; **2**: 601-605
14. Versnel MA, Claesson-Welsh L, Hammacher A, Bouts MJ, Van der Kwast ThH, Eriksson A, Willemsen R, Weima SM, Hoogsteden HC, Hagemeijer A, Heldin C-H. Human malignant mesothelioma cell lines express PDGF β-receptors whereas cultured normal mesothelial cells express predominantly PDGF α-receptors. *Oncogene* 1991; **6**: 2005-2011
15. Versnel MA, Bouts MJ, Hoogsteden HC, Van der Kwast ThH, Delahaye M, Hagemeijer A. Establishment of human malignant mesothelioma cell lines. *Int J Cancer* 1989; **44**: 256-260
16. Langerak AW, Dirks RPH, Versnel MA. Splicing of the platelet-derived growth factor A-chain mRNA in human malignant mesothelioma cell lines and regulation of its expression. *Eur J Biochem* 1992; **208**: 589-596
17. Versnel MA, Haarbrink M, Langerak AW, De Laat PAJM, Hagemeijer A, Van der Kwast ThH, Van den Berg-Bakker LAM, Schrier PI. Human ovarian tumors from epithelial origin express PDGF *in vitro* and *in vivo*. *Cancer Genet Cytogenet* 1994; **73**: 60-64
18. Ramaekers FCS, Huysmans A, Moesker O, Kant A, Jap PHK, Herman C, Vooys GP. Monoclonal antibody to keratin filaments, specific for glandular epithelia and their tumors. Use in surgical pathology. *Lab Invest* 1983; **49**: 353-361
19. Stahel RA, O'Hara CJ, Waibel R, Martin A. Monoclonal antibodies against mesothelial membrane antigen discriminate between malignant mesothelioma and lung adenocarcinoma. *Int J Cancer* 1988; **41**: 218-223
20. Shiraishi T, Morimoto S, Itoh K, Sato H, Sugihara K, Onishi T, Ogihara T. Radioimmunoassay of human platelet-derived growth factor using monoclonal antibody towards a synthetic 73-97 fragment of its B-chain. *Clin Chim Acta* 1989; **184**: 65-74
21. Eccleston PA, Funa K, Heldin C-H. Expression of platelet-derived growth factor (PDGF) and PDGF α- and ß-receptors in the peripheral nervous system: an analysis of sciatic nerve and dorsal root ganglia. *Dev Biol* 1993; **155**: 459-470
22. Kelly JD, Haldeman BA, Grant FJ, Murray MJ, Seifert RA, Bowen-Pope DF, Cooper JA, Kazlauskas A. Platelet-derived growth factor (PDGF) stimulates PDGF receptor subunit dimerization and intersubunit *trans*-phosphorylation. *J Biol Chem* 1991; **266**: 8987-8992
23. Connell ND, Rheinwald JG. Regulation of the cytoskeleton in mesothelial cells: reversible loss of keratin and increase in vimentin during rapid growth in culture. *Cell* 1983; **34**: 245-253
24. Manning LS, Whitaker D, Murch AR, Garlepp MJ, Davis MR, Musk AW, Robinson BWS. Establishment and characterization of five human malignant mesothelioma cell lines derived from pleural effusions. *Int J Cancer* 1991; **47**: 285-290
25. Lanfrancone L, Boraschi D, Ghiara P, Falini B, Grignani F, Peri G, Mantovani A, Pelicci PG. Human peritoneal mesothelial cells produce many cytokines (granulocyte colony-stimulating factor [CSF], granulocyte-monocyte-CSF, macrophage-CSF, interleukin-1 [IL-1], and IL-6) and are activated and stimulated to grow by IL-1. *Blood* 1992; **80**: 2835-2842
26. Garlepp MJ, Christmas TI, Manning LS, Mutsaers SE, Dench J, Leong C, Robinson BWS. The role of platelet-derived growth factor in the growth of human malignant mesothelioma. *Eur Resp Rev* 1993; **3**: 189-191

27. Ramael M, Buysse C, Van de Bossche J, Segers K, Van Marck E. Immunoreactivity for the β chain of the platelet-derived growth factor receptor in malignant mesothelioma and non-neoplastic mesothelium. *J Pathol* 1992; 167: 1-4
28. Terracio L, Rönnstrand L, Tingström A, Rubin K, Claesson-Welsh L, Funa K, Heldin C-H. Induction of platelet-derived growth factor receptor expression in smooth muscle cells and fibroblasts upon tissue culturing. *J Cell Biol* 1988; 107: 1947-1957
29. Antoniades HN, Galanopoulos T, Neville-Golden J, O'Hara CJ. Malignant epithelial cells in primary human lung carcinomas coexpress *in vivo* platelet-derived growth factor (PDGF) and PDGF receptor mRNAs and their protein products. *Proc Natl Acad Sci USA* 1992; 89: 3942-3946
30. Hermanson M, Funa K, Hartman M, Claesson-Welsh L, Heldin C-H, Westermark B, Nistér M. Platelet-derived growth factor and its receptors in human glioma tissue: expression of messenger RNA and protein suggests the presence of autocrine and paracrine loops. *Cancer Res* 1992; 52: 3213-3219
31. Plate KH, Breier G, Farrell CL, Risau W: Platelet-derived growth factor receptor-ß is induced during tumor development and upregulated during tumor progression in endothelial cells in human gliomas. *Lab Invest* 1992; 67: 529-534
32. Forsberg K, Valyi-Nagy I, Heldin C-H, Herlyn M, Westermark B. Platelet-derived growth factor (PDGF) in oncogenesis: Development of a vascular connective tissue stroma in xenotransplanted human melanoma producing PDGF-BB. *Proc Natl Acad Sci USA* 1993; 90: 393-397

4

REGULATION OF PDGF CHAIN AND RECEPTOR EXPRESSION IN MESOTHELIAL CELL LINES

CHAPTER 4.1

Splicing of the platelet-derived-growth-factor A-chain mRNA in human malignant mesothelioma cell lines and regulation of its expression

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Platelet-derived-growth-factor (PDGF) A-chain transcripts differing in the presence or absence of an alternative exon-derived sequence have been described. In some publications, the presence of PDGF A-chain transcripts with this exon-6-derived sequence was suggested to be tumour specific. However, in this paper it was shown by reverse-transcription polymerase-chain-reaction (PCR) analysis that both normal mesothelial cells and malignant mesothelioma cell lines predominantly express the PDGF A-chain transcript without the exon-6-derived sequence. This sequence encodes a cell-retention signal, which means that the PDGF A-chain protein is most likely to be secreted by both cell types. In cultured normal mesothelial cells, the secreted PDGF A-chain protein might be involved in autocrine growth stimulation via PDGF α receptors. However, human malignant mesothelioma cell lines only possess PDGF β receptors. If this also holds true *in vivo*, the PDGF A-chain protein produced and secreted by malignant mesothelial cells might have a paracrine function.

In a previous paper, we described elevated expression of the PDGF A-chain transcript in human malignant mesothelioma cell lines, compared to normal mesothelial cells. In this paper, the possible reason for this elevation was studied. First, alterations at the genomic level were considered, but cytogenetic and Southern-blot analysis revealed neither consistent chromosomal aberrations, amplification nor structural rearrangement of the PDGF A-chain gene in the malignant cells. Possible differences in transcription rate of the PDGF A-chain gene, and stability of the transcript between normal and malignant cells, were therefore studied. The presence of a protein-synthesis inhibitor, cycloheximide, in the culture medium did not significantly influence the PDGF A-chain mRNA level in normal mesothelial and malignant mesothelioma cell lines. Furthermore, nuclear run-off analysis showed that nuclear PDGF A-chain mRNA levels varied in both cell types to the same extent as the levels observed in Northern blots. Taken together, this suggests that increased transcription is the most probable mechanism for the elevated mRNA level of the PDGF A-chain gene in human malignant mesothelioma cell lines.

Platelet-derived growth factor (PDGF) is composed of homodimers or heterodimers of two polypeptide chains, denoted A and B. These polypeptides are encoded by two distinct genes, which show a high degree of similarity (Betsholtz et al., 1986). All three dimeric combinations (AA, AB and BB) have been identified (Stroobant and Waterfield, 1984; Heldin et al., 1986; Hammacher et al., 1988). Based on ligand-binding and cross-competition analysis, two different PDGF receptors, α and β, have been described (Hart et al., 1988; Heldin et al., 1988). The structure of these receptors is quite similar, showing most variation in the extracellular binding domain (Claesson-Welsh et al., 1988, 1989; Matsui et al., 1989). The two receptor subtypes show different affinities for the dimeric PDGF isoforms. The PDGF α receptor binds all three forms with high affinity, whereas the β-receptor subtype only binds PDGF BB with high affinity (Claesson-Welsh et al., 1988, 1989; Hammacher et al., 1989; Seifert et al., 1989). The reports on the ability of PDGF AB to bind to PDGF β receptors are conflicting (Hammacher et al., 1989; Seifert et al., 1989; Grotendorst et al., 1991; Heidaran et al., 1991; Drozdoff and Pledger, 1991).

Although originally isolated from blood platelets, PDGF seems also to be produced by several other cell types, e.g. endothelial cells and smooth muscle cells. Furthermore, expression of one or both of the PDGF chains has been reported for a variety of tumour cell types, such as osteosarcoma and glioblastoma (reviewed in Heldin and Westermark, 1990; Raines et al., 1990).

Human malignant mesothelioma is a tumour of mesodermal origin and is predominantly found in the pleura. The incidence of malignant mesothelioma is strongly associated with asbestos exposure (Wagner et al., 1960). Malignant mesothelioma is thought to develop from cells of mesothelial

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Abbreviations. PDGF, platelet-derived growth factor; PCR, polymerase chain reaction.

*Enzymes. Hin*dIII (EC 3.1.23.20); *Xba*I restriction endonuclease (EC 3.1.23.41); RNAse A (EC 3.1.27.5); RNase-free DNAse I (EC 3.1.21.1); avian myoblastoma virus reverse transcriptase (EC 2.7.7.49); *Taq* polymerase (EC 2.7.7.7); T4 polynucleotide kinase (EC 2.7.1.78).

origin. Expression of PDGF B-chain and PDGF β-receptor transcripts in a panel of human malignant mesothelioma cell lines was reported earlier, whereas normal mesothelial cell lines express no PDGF B-chain mRNA and little or no PDGF β-receptor mRNA (Gerwin et al., 1987; Versnel et al., 1988, 1991). Normal mesothelial cell lines, in contrast, were found to express PDGF α-receptor mRNA, which could not be detected in mesothelioma cell lines (Versnel et al., 1991). PDGF A-chain mRNA was detected both in normal and malignant mesothelial cell lines, but the latter clearly showed an elevated expression of this gene (Gerwin et al., 1987; Versnel et al., 1988).

In several reports, alternative splicing of exon 6, resulting in an extra 69-bp internal region, was shown for the PDGF A-chain mRNA (Collins et al., 1987; Rorsman et al., 1988; Bonthron et al., 1988; Matoskova et al., 1989). Since in some publications expression of the transcript with the exon-6-derived sequence was suggested to be tumour specific (Collins et al, 1987; Rorsman et al., 1988), the presence of this extra exon in the A-chain mRNA was studied in both normal and malignant mesothelial cell lines. We found no increased use of the exon-6 element in transcripts produced by malignant versus normal mesothelial cell lines. Furthermore, in this paper, the possible cause of the observed elevated expression of the PDGF A-chain gene in malignant mesothelioma cell lines was also studied. Increased transcription was found to be the most probable mechanism for this elevation.

MATERIALS AND METHODS

Cell lines, conditions of growth and cytogenetic analysis

Experiments were performed using the human malignant mesothelioma cell lines Mero-14, Mero-25, Mero-41, Mero-48b, Mero-48c, Mero-72, Mero-82, Mero-83, Mero-84, Mero-95 and Mero-96 (Versnel et al., 1989) and the normal mesothelial cell lines NM-1, NM-2, NM-5, NM-6, NM-7, NM-9, NM-10, NM-11, NM-12 and NM-13. Cell lines were routinely cultured as described by Versnel et al. (1989). When indicated, cycloheximide (Sigma, St. Louis, MO) was added to the medium at a concentration of 10 μg/ml for 2 h, and actinomycin D (Sigma) at 5 μg/ml for 1–4 h. Cytogenetic analysis was performed as described by Versnel et al. (1988).

Reverse-transcription PCR analysis and oligonucleotide hybridization

RNA was isolated as described by Versnel et al. (1988). Chromosomal DNA was removed by treating 10 μg total RNA for 1 h at 37°C with 10 μg RNAse-free DNAse I (BRL, Gaithersburg, MD). After ethanol precipitation, 1 μg DNAse-I-treated RNA was used in a reverse transcriptase reaction, modified from Krug and Berger (1987). First 0.01 U $(dT)_{15}$ (Pharmacia, Uppsala, Sweden) was added to the RNA to give a final volume of 14 μl, and the mixture was heated for 3 min at 85°C. The oligo(dT)-primed RNA was then added to a mixture containing 50 mM Tris/HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 1 μg/ml bovine serum albumin, 1 mM dNTP, 4 mM sodium pyrophosphate, 40 U RNAsin (Promega, Madison, WI) and 5 U avian-myoblastoma-virus reverse transcriptase (Boehringer Mannheim, FRG). This mixture was incubated for 1 h at 39°C. Of this cDNA mixture, 25% was used in a PCR reaction. cDNA was mixed with 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (mass/vol.) gelatin, 0.2 mM dNTP, PDGF A-chain sense and antisense primers (A_{260} = 0.2) and 1 U *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT). The primers used were the same as in Matoskova et al. (1989). 35 cycles (1 min at 94°C for denaturation, 2 min at 55°C for annealing and 3 min at 72°C for primer extension) of amplification were performed, using the Perkin-Elmer Cetus DNA thermal cycler.

The PCR reaction mixture was analysed on a 1.5% (mass/vol.) agarose gel. After 10 min depurination (0.25 M HCl), two 15-min periods of denaturation (1.5 M NaCl, 0.5 M NaOH) and two 20-min periods of neutralization (1.5 M NaCl, 0.5 M Tris/HCl, pH 7), PCR fragments were blotted onto Hybond-N (Amersham, UK) in 1.5 M NaCl, 150 mM trisodium citrate, pH 7, and were immobilized by ultraviolet cross-linking. A 53-bp oligonucleotide primer complementary to a part of the PDGF A-chain exon-6 sequence, [5′-d(GGTGGGTTTTAACCTTTTCTTTCCGTTTTTTAC-CTGACTCCCTAGGCCTTC)-3′; Fig. 1A] was used for oligonucleotide hybridization of the filter. The oligomer (12.5 pmol) was end-labeled with 40 μCi [γ-^{32}P]ATP in a reaction mixture containing 50 mM Tris/HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, 0.1 mM EDTA and 10 U T4 polynucleotide kinase (Pharmacia) for 30 min at 37°C. Oligonucleotide hybridization was performed at 65°C for 2 h and the filter was washed twice for 30 min in 0.1% SDS, 0.9 M NaCl, 50 mM NaH_2PO_4 and 5 mM EDTA at 65°C. The filter was rehybridized with the 1.3-kb *Eco*RI PDGF A-chain fragment (Figs 1A and 4A), which was labeled with ^{32}P by random-primer labeling. Hybridization conditions with this probe were the same as described for Southern-blot analysis (Versnel et al., 1988). Autoradiography was performed with Fuji-RX films at room temperature for 10–20 min.

Northern-blot and Southern-blot analyses

RNA isolation and Northern blotting were performed as described by Versnel et al. (1988). Filters were washed in 45 mM NaCl and 4.5 mM trisodiun citrate, pH 7.0, at 42°C. DNA isolation and Southern blotting were also described by Versnel et al. (1988). Filters were exposed to Fuji-RX films.

Nuclear run-off assay

Cells were cultured as described. Nuclei were isolated from 10^8 cells, essentially according to Zenke et al. (1988) except for the presence of 0.5% Nonidet P-40 in lysis buffer. From each cell line, $1-2\times10^7$ nuclei were used for a nuclear run-off assay, adapted from Linial et al. (1985). The ultimate concentration of Tris/HCl, pH 8.0, in the run-off buffer was 6 mM, and 140 μCi [α-^{32}P]UTP was added. Transcripts were synthesized at 30°C for 20 min, followed by a 5-min DNAse I (10 μg) digestion at 30°C. After centrifugation, the nuclear RNA pellet was resuspended in hybridization buffer containing 45% (by vol.) formamide, 0.2 M sodium phosphate, pH 7.2, 1 mM EDTA, 7% SDS and 250 μg/ml yeast RNA.

Plasmid DNA was spotted on to nitrocellulose filters with a slot-blot apparatus (Schleicher and Schuell, Dassel, FRG) and immobilized for 2 h at 80°C. Subsequently, the filters were hybridized with the ^{32}P-labeled RNA for 2 days at 45°C. After hybridization, filters were washed for 3–4 h in 40 mM sodium phosphate, pH 7.2, and 1% SDS at 65°C, interrupted by a washing step of 30 min at 37°C in 0.3 M NaCl and 30 mM trisodium citrate, pH 7.0, containing 5 μg/ml RNAse

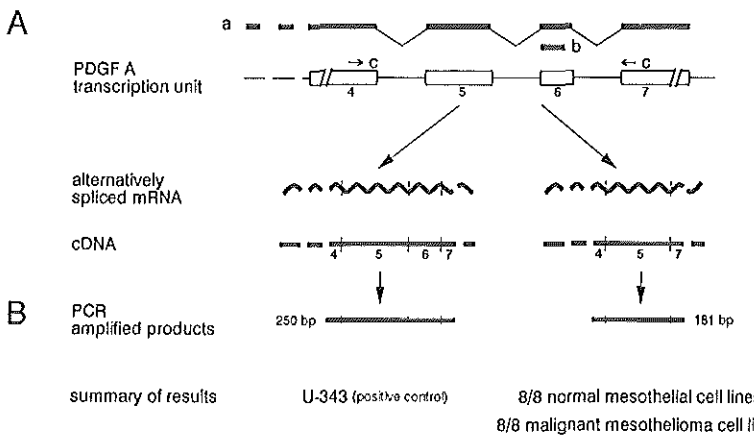

Fig. 1. Alternative splicing of the PDGF A-chain gene in normal and malignant mesothelial cell lines. (A) Part of the PDGF A-chain transcription unit with positions of PDGF A-chain probe (a), the exon-6-specific oligomer (b) and the primers used for PCR (c). The two alternatively spliced mRNA (including exon 6 or not) can be reverse-transcribed to cDNA. (B) PCR-mediated amplification of cDNA results in fragments of 250 bp, as found in the control cell line U-343, or 181 bp, observed in eight normal and eight malignant mesothelial cell lines.

A. Autoradiography was performed at −80°C with Kodak XAR films.

Probes

The PDGF A-chain probe (Figs 1A and 4A) was an *Eco*RI fragment of 1.3 kb (Betsholtz et al., 1986). The glyceraldehyde-3-phosphate-dehydrogenase probe was a 0.7-kb *Eco*RI – *Pst*I fragment (Benham et al., 1984). The T-cell-receptor Cγ probe was a 0.4-kb *Bam*HI fragment (van Dongen and Wolvers-Tettero, 1991). For the run-off analysis, the above-mentioned PDGF A-chain fragment was used, subcloned in pUC. pAct (Dodemont et al., 1982) was used to detect actin expression in this assay.

RESULTS

PDGF A-chain exon-6-derived sequences are not expressed in normal and malignant mesothelial cell lines

For reverse-transcription PCR analysis of the PDGF A-chain mRNA, total RNA of several malignant mesothelioma (Mero-14, Mero-25, Mero-48c, Mero-72, Mero-83, Mero-84, Mero-95 and Mero-96) and normal mesothelial (NM-1, NM-2, NM-6, NM-7, NM-10, NM-11, NM-12 and NM-13) cell lines was reverse-transcribed. cDNA was amplified by PCR. Using the PDGF-A-chain-specific oligonucleotide primers, which were described by Matoskova et al. (1989), fragments of 250 bp (including the exon-6-derived sequence) or 181 bp (without exon-6-derived cDNA) were amplified (Fig. 1). The PDGF A-chain cDNA from glioma cell line U-343 was used as a positive control for the presence of the exon-6-derived sequence (Matoskova et al., 1989). Amplification of this cDNA with these primers resulted in a DNA fragment of 250 bp, as shown in Figs 1 – 3. Non-reverse-transcribed RNA

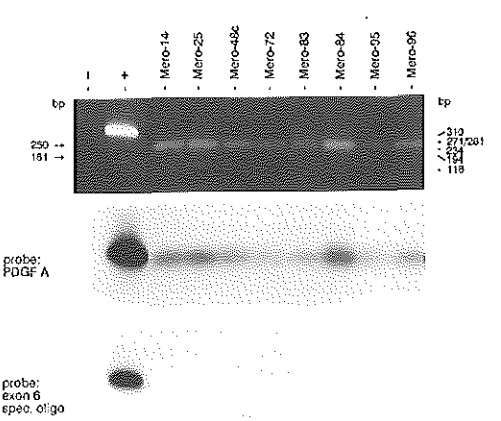

Fig. 2. Reverse-transcription PCR analysis with RNA from malignant mesothelioma cell lines Mero-14, Mero-25, Mero-48c, Mero-72, Mero-83, Mero-84, Mero-95 and Mero-96 on an ethidium-bromide-stained agarose gel. cDNA from glioma cell line U-343 was used as a positive control (+), and RNA from normal mesothelial cell line NM-2 as a negative control (−) for the PCR reaction. After blotting of the gel, the filter was hybridized to a PDGF A-chain probe and to an exon-6-specific oligomer (spec. oligo), as described in the legend to Fig. 1A.

of cell line NM-2 was used as a negative control for the PCR reaction. In the normal, as well as in the malignant, mesothelial cell lines studied, PCR-mediated amplification resulted in a predominant band of 181 bp on ethidium-bromide-stained agarose gels (Figs 1 – 3). Only in some cases was a faint band of 250 bp detected (data not shown). Band size was confirmed by electrophoresis on a 10% polyacrylamide

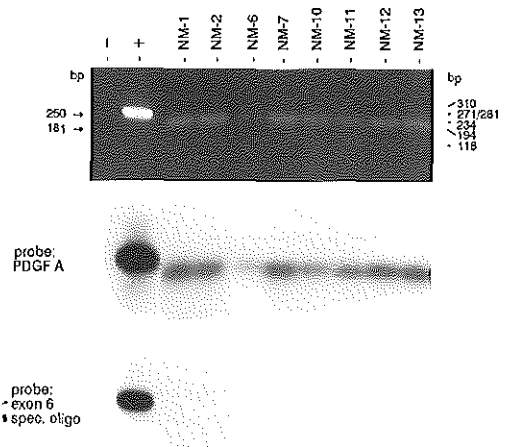

Fig. 3. Reverse-transcription PCR analysis with RNA from normal mesothelial cell lines NM-1, NM-2, NM-6, NM-7, NM-10, NM-11, NM-12 and NM-13 on an ethidium-bromide-stained agarose gel. cDNA from glioma cell line U-343 was used as a positive control (+) and RNA from normal mesothelial cell line NM-2 as a negative control (−) for the PCR reaction. After blotting of the gel, the filter was hybridized to a PDGF A-chain probe and to an exon-6-specific oligomer (spec. oligo), as described in the legend to Fig. 1.

gel (data not shown). The nature of the bands on agarose gels was studied by hybridization to an exon-6-specific oligonucleotide primer and to the PDGF A-chain cDNA probe. As expected, the PDGF A-chain probe hybridized to the 181-bp and the 250-bp fragments, and not to the marker DNA (Figs 1–3). However, the exon-6-specific oligomer hybridized only to the 250-bp band (Figs 1–3), showing the absence of the exon-6-derived sequence in the 181-bp fragment. It was therefore shown by hybridization that normal and malignant mesothelial cell lines predominantly contain the 181-bp fragment variant of the PDGF A-chain. On longer exposure, however, in some cell lines faint bands of 250 bp were also seen. A summary of these data is given in Fig. 1B.

Malignant mesothelioma cell lines do not show structural rearrangements or amplification of the PDGF A-chain gene

As a possible reason for elevated PDGF A-chain mRNA expression in malignant mesothelioma cell lines, structural rearrangement or amplification of the PDGF A-chain gene were considered. *Xba*I/*Hin*dIII-digested DNA from the normal mesothelial cell line NM-6 and from the malignant mesothelioma cell lines Mero-14, Mero-25, Mero-41, Mero-48b and Mero-82 was used for Southern-blot analysis with a PDGF A-chain cDNA probe (Fig. 4A). *Xba*I/*Hin*dIII-digested DNA of these cell lines did not reveal structural rearrangements in the PDGF A-chain gene. In normal as well as malignant cells, a band of about 15.6 kb was detectable after *Hin*dIII digestion (Fig. 4B). On longer exposure, a second band of about 25 kb, probably corresponding to a fragment upstream of the 15.6-kb fragment, was detected in the investigated cell lines (Fig. 4B). The bands seen on Southern blots of *Xba*I-digested (data not shown) and *Hin*dIII-digested (Fig. 4B)

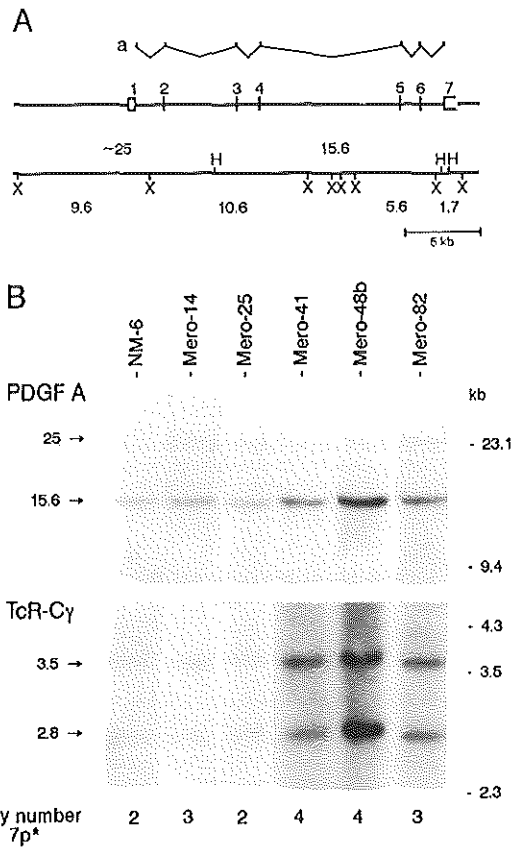

Fig. 4. Southern-blot analysis of the PDGF A-chain gene in normal and malignant mesothelial cell lines. (A) Part of the PDGF A-chain locus with PDGF A-chain cDNA probe (a), restriction sites for the enzymes *Xba*I (X) and *Hin*dIII (H) and exons numbered 1–7. Adapted from Bonthron et al. (1988). (B) Southern-blot analysis with DNA from normal mesothelial cell line NM-6 and malignant mesothelioma cell lines Mero-14, Mero-25, Mero-41, Mero-48b and Mero-82. DNA was hybridized to PDGF A-chain and T-cell-receptor (TcR) Cγ probes. *, number of normal and rearranged copies of chromosome 7p.

DNA correspond to DNA fragments in the PDGF A-chain locus, which are indicated in Fig. 4A.

The same filters were subsequently hybridized with a T-cell-receptor Cγ probe, since this locus, like the PDGF A-chain, has been mapped to chromosome 7p. This resulted in bands of 2.8 bp and 3.5 kb in all cell lines investigated after *Hin*dIII digestion (Fig. 4B). The intensities of the bands correlated with those after PDGF A-chain hybridization, which suggests that there is no amplification of the PDGF A-chain gene in malignant mesothelioma cell lines. Correlation of the intensities of the bands from the various cell lines with the copy number of chromosome 7p (Fig. 4B) showed that variation in

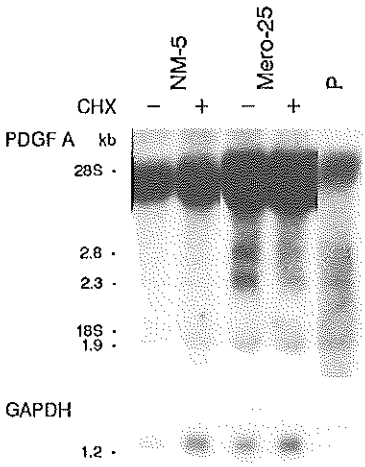

Fig. 5. Northern-blot analysis with 25 μg total RNA from placenta (P), normal mesothelial cell line NM-5 and malignant mesothelioma cell line Mero-25, cultured in the presence (+) or absence (−) of 10 μg/ml cycloheximide (CHX). RNA was hybridized to PDGF A-chain and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) probes.

copy number is the major cause of the difference in band intensity seen in the various lanes.

Increased mRNA stability does not seem to be the cause of elevated PDGF A-chain levels in malignant mesothelioma cell lines

Elevated expression of mRNA on Northern blots can, in general, be accounted for by increased transcription or by increased stability of the transcript in the cytoplasm. To discern between these possibilities, the human malignant mesothelioma cell lines Mero-25, Mero-48c and Mero-82 and the normal mesothelial cell lines NM-5, NM-7 and NM-9 were cultured in the absence or presence of a protein synthesis inhibitor, cycloheximide, for 2 h. RNA isolated from these cell lines was analyzed for the expression of PDGF A-chain mRNA. Placental RNA was used as a positive control. Addition of cycloheximide did not affect levels of PDGF A-chain transcripts (2.8, 2.3 and 1.9 kb) significantly in the three normal or in the three malignant mesothelial cell lines investigated. This is shown for the normal mesothelial cell line NM-5 and the malignant mesothelioma cell line Mero-25 (Fig. 5). Approximately equal amounts of RNA were loaded in each lane, which was demonstrated by rehybridization of the filter with glyceraldehyde-3-phosphate dehydrogenase (Fig. 5).

Nuclear RNA expression levels of the PDGF A-chain were studied in the malignant mesothelioma cell lines Mero-25 and Mero-82 and in the normal mesothelial cell lines NM-5 and NM-7. By nuclear run-off analysis, nuclear PDGF A-chain RNA levels were found to be increased in the two malignant mesothelioma cell lines, compared to the two normal mesothelial cell lines. In Fig. 6, this is demonstrated for NM-5 and Mero-25. This increase was comparable to the earlier observed increase on Northern blots between the two cell types

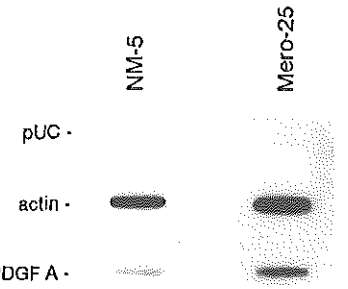

Fig. 6. Nuclear run-off analysis with ^{32}P-labeled nuclear RNA from normal mesothelial cell line NM-5 and malignant mesothelioma cell line Mero-25, on nitrocellulose blots containing the plasmids pUC, pUC plus actin and pUC plus PDGF A chain at the indicated positions.

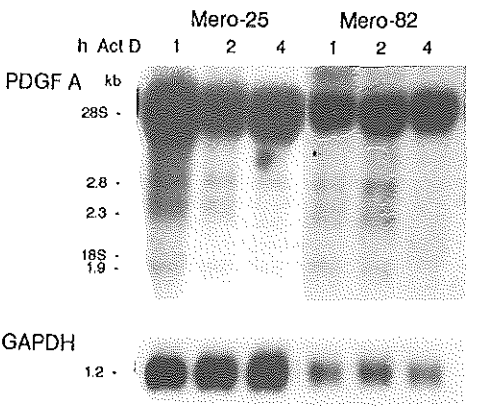

Fig. 7. Northern-blot analysis with 25 μg total RNA from malignant mesothelioma cell lines Mero-25 and Mero-82, cultured in the presence of 5 μg/ml actinomycin D (Act D) for the indicated time. RNA was hybridized to PDGF A-chain and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) probes.

(data not shown). Concomitant analysis of the constitutively transcribed actin gene revealed that the same amounts of nuclear RNA were analyzed in the various cells. Non-specific hybridization to the pUC vector was not observed (Fig. 6). Thus, the difference in PDGF A-chain mRNA expression between normal and malignant mesothelial cell lines, as detected by Northern-blot analysis, was also reflected in the amount of nuclear transcripts.

Northern-blot analysis of RNA from the malignant mesothelioma cell lines Mero-25 and Meso-82, cultured for various times with the transcription inhibitor actinomycin D, revealed a sudden decrease in PDGF A-chain mRNA expression, after 2–4 h treatment with actinomycin D, which was not accompanied by a similar decrease in the glyceraldehyde-3-phosphate-dehydrogenase mRNA expression (Fig. 7). The level of expression after 1 h treatment with actinomycin D was comparable to the level found in untreated cells of Mero-25 and Mero-82 (data not shown). These results suggest a half-life of less than 4 h for the PDGF A-chain mRNA in these cell lines. Unfortunately, we did not succeed in isolating

enough RNA from actinomycin-D-treated normal mesothelial cells to study the half-life of the A-chain transcript in normal cells.

DISCUSSION

Alternatively spliced PDGF A-chain transcripts, which differ in the presence or absence of exon-6-derived sequences, have been described. In some publications, transcripts including this sequence were thought to be tumour specific (Collins et al., 1987; Rorsman et al., 1988). The results in this study suggest that in both normal and malignant mesothelial cell lines, PDGF A-chain mRNA without exon 6 was the predominant transcript. In some cell lines, only tiny amounts of the alternative mRNA, which includes the exon-6-derived sequence, were observed. Thus, the results obtained in mesothelial cells are consistent with earlier described data by Matoskova et al. (1989), who suggested that usage of exon-6 was not tumour specific. Of the two proteins formed, the PDGF A-chain protein encoded by the smaller transcript is efficiently secreted (Östman et al., 1991). The protein encoded by the larger transcript, however, is retained in the cell due to a cell-retention signal in the exon-6-derived domain of this protein (Maher et al., 1989; Östman et al., 1991). Thus, in both normal and malignant mesothelial cells, secretable PDGF A-chain protein is most often encountered.

Cultured normal human mesothelial cells predominantly express PDGF α receptors (Versnel et al., 1991). Therefore, secreted PDGF A-chain protein could have an autocrine function in normal mesothelial cells *in vitro*. Whether this also holds true *in vivo* remains to be determined, since PDGF receptors are easily induced in culture (Terracio et al., 1988), and no data are currently available about PDGF α receptors in freshly isolated normal mesothelial cells. Human malignant mesothelioma cell lines, however, were shown to produce only PDGF β receptors (Versnel et al., 1991). The secretable PDGF A-chain protein, produced by malignant mesothelioma cell lines, might therefore act as a paracrine growth factor. Possible target cells for this paracrine growth activity could be mesothelial cells, fibroblasts or smooth muscle cells.

PDGF A-chain mRNA levels were earlier shown to be elevated in human malignant mesothelioma cell lines, compared to cultured normal mesothelial cells (Gerwin et al., 1987; Versnel et al., 1988). In this paper, the possible reason for this elevation was studied. The culture conditions for normal and malignant mesothelial cell lines are almost identical, except for the addition of epidermal growth factor and hydrocortisone to the culture medium of normal mesothelial cells. Recently, PDGF A-chain mRNA and protein expression were reported to be decreased in hepatoma cells cultured in the presence of the glucocorticoid dexamethasone (Haraguchi et al., 1991). Malignant mesothelioma cell lines cultured in the presence of epidermal growth factor and hydrocortisone, alone or in combination, however, showed a similar level of PDGF A-chain mRNA to that found in the absence of these agents (data not shown). Moreover, the absence of epidermal growth factor or hydrocortisone in the culture medium of normal mesothelial cells did not result in an increase in PDGF A-chain mRNA to the level seen in malignant cells (data not shown).

Alterations at the genomic level were considered a possible reason for elevated expression of the PDGF A-chain gene. However, by cytogenetic analysis, the copy number of chromosome 7 was previously shown to be variable between the ten different human malignant mesothelioma cell lines, but not to be related to the PDGF A-chain mRNA level. Moreover, no consistent specific marker of chromosome 7, which might be involved in PDGF A-chain expression, could be found in these cell lines (Versnel et al., 1988). In this study, Southern-blot analysis did not reveal amplification of the PDGF A-chain gene or structural rearrangements in the investigated region.

Based on these observations, increased transcription and increased stability of the transcript were studied as the possible mechanism for elevated PDGF A-chain mRNA expression in human malignant mesothelioma cell lines. Since the protein-synthesis inhibitor cycloheximide did not significantly influence the expression of the PDGF A-chain transcript in normal and malignant mesothelial cells, *de-novo*-synthesized proteins do not seem to affect expression of this gene in these cell types.

Furthermore, the level of nuclear PDGF A-chain transcripts in malignant mesothelioma cells compared with normal mesothelial cells was elevated to the same extent as the steady-state mRNA level in Northern blots. Taken together, these results point towards increased transcription as the most probable cause for the elevated expression in malignant mesothelioma cell lines. Differences in mRNA stability are probably less important in this respect.

Many different regulators of PDGF A-chain mRNA expression have been found in various cell types *in vitro*. PDGF A-chain expression in microvascular endothelial cells was shown to be increased by transforming growth factor β and phorbol ester (Starksen et al., 1987), and by acidic fibroblast growth factor, interleukin-1, interleukin-6, tumour necrosis factor α and phorbol ester in human endothelial cells (Gay and Winkles, 1990). In smooth muscle cells, PDGF A-chain mRNA was positively modulated by acidic fibroblast growth factor, tumour necrosis factor α, transforming growth factor β, phorbol ester and serum (Winkles and Gay, 1991), and in fibroblasts by PDGF and interleukin-1 (Paulsson et al., 1987; Raines et al., 1989). Some of these regulators are thought to act by transcriptionally activating the PDGF A-chain gene. One or more might also be involved in modulation of PDGF A-chain gene expression in malignant mesothelioma cell lines. However, this remains to be demonstrated in further experiments.

A half-life of less than 4 h was observed for PDGF A-chain mRNA in human malignant mesothelioma cell lines. This is similar to the previously described half-life of about 4 h in rat aortic smooth muscle cells (Majesky et al., 1988) and differs slightly from the half-life of 2.4 h in human umbilical-vein endothelial cells (Gay and Winkles, 1991).

In conclusion, in this paper, we demonstrate that both normal and malignant human mesothelial cells predominantly produce PDGF A-chain transcripts without exon-6-derived sequences. This means that normal and malignant mesothelial cells probably secrete PDGF A-chain proteins, due to the absence of the sequence encoding a cell-retention signal in the transcript. We suggest that the PDGF A-chain protein, produced and secreted by malignant mesothelioma cell lines, probably has a paracrine function. Furthermore, we also demonstrate that the elevated PDGF A-chain mRNA expression in human malignant mesothelioma cell lines is presumably caused by an increased transcription of this gene. The causes of this increased transcription rate will be further investigated in the future by localisation of regulatory regions in the PDGF A-chain gene and characterisation of the possible factor(s) acting on them.

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REFERENCES

Benham, F. J., Hodgkinson, S. & Davies, K. E. (1984) A glyceraldehyde-3-phosphate dehydrogenase pseudogene on the short arm of the human X-chromosomes defines a multigene family, *EMBO J. 3*, 2635–2640.

Betsholtz, C., Johnsson, A., Heldin, C.-H., Westermark, B., Lind, P., Urdea, M. S., Eddy, R., Shows, T. B., Philpott, K., Mellor, A. L., Knott, T. J. & Scott, J. (1986) cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumor cell lines, *Nature 320*, 695–699.

Bonthron, D. T., Morton, C. C., Orkin, S. H. & Collins, T. (1988) Platelet-derived growth factor A-chain: gene structure, chromosomal location, and basis for alternative mRNA splicing, *Proc. Natl Acad. Sci. USA 85*, 1492–1496.

Claesson-Welsh, L., Eriksson, A., Morén, A., Severinsson, L., Ek, B., Östman, A., Betsholtz, C. & Heldin, C.-H. (1988) cDNA cloning and expression of human platelet-derived growth factor PDGF) receptor specific for B-chain-containing PDGF molecules, *Mol. Cell. Biol. 8*, 3476–3486.

Claesson-Welsh, L., Eriksson, A., Westermark, B. & Heldin, C.-H. (1989) cDNA cloning and expression of the human A type PDGF receptor establishes structural similarity to the B type PDGF receptor, *Proc. Natl Acad. Sci. USA 86*, 4917–4921.

Collins, T., Bonthron, D. T. & Orkin, S. H. (1987) Alternative splicing affects function of encoded platelet-derived growth factor A chain, *Nature 328*, 621–624.

Dodemont, H. J., Soriano, P., Quax, W. J., Ramaekers, F., Lenstra, J. A., Groenen, M. A., Bernardi, G. & Bloemendal, H. (1982) The genes coding for the cytoskeletal proteins actin and vimentin in warm-blooded vertebrates, *EMBO J. 1*, 167–171.

Drozdoff, V. & Pledger, W. J. (1991) Cellular response to platelet-derived growth factor (PDGF)-AB after down-regulation of PDGF α-receptors, *J. Biol. Chem. 266*, 17 165–17 172.

Gay, C. G. & Winkles, J. A. (1990) Heparin-binding growth factor-1 stimulation of human endothelial cells induces platelet-derived growth factor A-chain gene expression, *J. Biol. Chem. 265*, 3284–3292.

Gay, C. G. & Winkles, J. A. (1991) The half-lives of platelet-derived growth factor A- and B-chain mRNAs are similar in endothelial cells and unaffected by heparin-binding growth factor-1 or cycloheximide, *J. Cell. Physiol. 147*, 121–127.

Gerwin, B. I., Lechner, J. F., Reddel, R. R., Roberts, A. B., Robbins, K. C., Gabrielson, E. W. & Harris, C. C. (1987) Comparison of production of transforming growth factor-beta and platelet-derived growth factor by normal human mesothelial cells and mesothelioma cell lines, *Cancer Res. 47*, 6180–6184.

Grotendorst, G. R., Igarashi, A., Larson, R., Soma, Y. & Charette, M. (1991) Differential binding, biological and biochemical actions of recombinant PDGF AA, AB, BB molecules on connective tissue cells, *J. Cell. Physiol. 149*, 235–243.

Hammacher, A., Hellman, U., Johnsson, A., Östman, A., Gunnarson, K., Westermark, B., Wasteson, Å. & Heldin, C.-H. (1988) A major part of platelet-derived growth factor purified from human platelets is a heterodimer of one A and one B chain, *J. Biol. Chem. 263*, 16 493–16 498.

Hammacher, A., Mellström, K., Heldin, C.-H. & Westermark, B. (1989) Isoform-specific induction of actin reorganization by platelet-derived growth factor suggests that the functionally active receptor is a dimer, *EMBO J. 8*, 2489–2495.

Haraguchi, T., Alexander, D. B., King, D. S., Edwards, C. P. & Firestone, G. L. (1991) Identification of the glucocorticoid suppressible mitogen from rat hepatoma cells as an angiogenic platelet-derived growth factor A-chain homodimer, *J. Biol. Chem. 266*, 18 299–18 307.

Hart, C. E., Forstrom, J. W., Kelly, J. D., Seifert, R. A., Smith, R. A., Ross, R., Murray, M. & Bowen-Pope, D. F. (1988) Two classes of PDGF receptors recognize different isoforms of PDGF, *Science 240*, 1529–1531.

Heidaran, M. A., Pierce, J. H., Yu, J.-C., Lombardi, D., Artrip, J. E., Fleming, T. P. Thomason, A. & Aaronson, S. A. (1991) Role of receptor heterodimer formation in β platelet-derived growth factor (PDGF) receptor activation by PDGF-AB, *J. Biol. Chem. 266*, 20 232–20 237.

Heldin, C.-H., Johnsson, A., Wennergren, S., Wernstedt, C., Betsholtz, C. & Westermark, B. (1986) A human osteosarcoma cell line secretes a growth factor structurally related to a homodimer of PDGF A-chains, *Nature 319*, 511–514.

Heldin, C.-H., Bäckström, G., Östman, A., Hammacher, A., Rönnstrand, L., Rubin, K., Nistér, M. & Westermark, B. (1988) Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types, *EMBO J. 7*, 1387–1393.

Heldin, C.-H. & Westermark, B. (1990) Platelet-derived growth factor: mechanism of action and possible *in vivo* function, *Cell Regul. 1*, 555–566.

Krug, M. S. & Berger, S. L. (1987) First-strand cDNA synthesis primed with oligo(dT), *Methods Enzymol. 152*, 316–325.

Linial, M., Gunderson, N. & Groudine, M. (1985) Enhanced transcription of *c-myc* in bursal lymphoma cells requires continuous protein synthesis, *Science 230*, 1126–1132.

Maher, D. W., Lee, B. A. & Donoghue, D. J. (1989) The alternatively spliced exon of the platelet-derived growth factor A chain encodes a nuclear targeting signal, *Mol. Cell. Biol. 9*, 2251–2253.

Majesky, M. W., Benditt, E. P. & Schwartz, S. M. (1988) Expression and developmental control of platelet-derived growth factor A-chain and B-chain/*sis* genes in rat aortic smooth muscle cells, *Proc. Natl Acad. Sci. USA 85*, 1524–1528.

Matoskova, B., Rorsman, F., Svensson, V. & Betsholtz, C. (1989) Alternative splicing of the platelet-derived growth factor A-chain transcript occurs in normal as well as tumor cells and is conserved among mammalian species, *Mol. Cell. Biol. 9*, 3148–3150.

Matsui, T., Heidaran, M., Mibi, T., Popescu, N., La Rochelle, W., Kraus, M., Pierce, J. & Aaronson, S. (1989) Isolation of a novel receptor cDNA establishes the existence of two PDGF receptor genes, *Science 243*, 800–803.

Östman, A., Andersson, M. Betsholtz, C., Westermark, B. & Heldin, C.-H. (1991) Identification of a cell retention signal in the B-chain of platelet-derived growth factor and in the long splice version of the A-chain, *Cell Regul. 2*, 503–512.

Paulsson, Y., Hammacher, A., Heldin, C.-H. & Westermark, B. (1987) Possible positive autocrine feedback in the prereplicative phase of human fibroblasts, *Nature 328*, 715–717.

Raines, E. W., Dower, S. K. & Ross, R. (1989) Interleukin-1 mitogenic activity for fibroblasts and smooth muscle cells is due to PDGF AA, *Science 243*, 393–396.

Raines, E. W., Bowen-Pope, D. F. & Ross, R. (1990) Peptide growth factors and their receptors, in *Handbook of experimental pharmacology* (Sporn, M. B. & Roberts, A. B., eds) vol. 95, part 1, pp. 173–262, Springer Verlag, Heidelberg.

Rorsman, F., Bywater, M., Knott, T. J., Scott, J. & Betsholtz, C. (1988) Structural characterization of the human platelet-derived growth factor A-chain cDNA and gene: alternative exon usage predicts two different precursor proteins, *Mol. Cell. Biol. 8*, 571–577.

Seifert, R. A., Hart, C. E., Phillips, P. E., Forstrom, J. W., Ross, R., Murray, M. J. & Bowen-Pope, D. F. (1989) Two different subunits associate to create isoform-specific platelet-derived growth factor receptors, *J. Biol. Chem. 264*, 8771–8778.

Starksen, N. F., Harsh IV, G. R., Gibbs, V. C. & Williams, L. T. (1987) Regulated expression of the platelet-derived growth factor A-chain gene in microvascular endothelial cells, *J. Biol. Chem. 262*, 14 381–14 384.

Stroobant, P. & Waterfield, M. D. (1984) Purification and properties of porcine platelet-derived growth factor, *EMBO J. 3*, 2963–2967.

Terracio, L., Rönnstrand, L., Tingström, A., Rubin, K., Claesson-Welsh, L., Funa, K. & Heldin, C.-H. (1988) Induction of platelet-derived growth factor receptor expression in smooth muscle cells and fibroblasts upon tissue culturing, *J. Cell Biol. 107*, 1947–1957.

Van Dongen, J. J. M. & Wolvers-Tettero, I. L. M. (1991) Analysis of immunoglobulin and T cell receptor genes, *Clin. Chim. Acta 198*, 1–174.

Versnel, M. A., Hagemeijer, A., Bouts, M. J., van der Kwast, T. H. & Hoogsteden, H. C. (1988) Expression of *c-sis* (PDGF B-chain) and PDGF A-chain genes in ten human malignant mesothelioma cell lines derived from primary and metastatic tumors, *Oncogene 2*, 601–605.

Versnel, M. A., Bouts, M. J., Hoogsteden, H. C., van der Kwast, T. H., Delahaye, M. & Hagemeijer, A. (1989) Establishment of human malignant mesothelioma cell lines, *Int. J. Cancer 44*, 256–260.

Versnel, M. A., Claesson-Welsh, L., Hammacher, A., Bouts, M. J., van der Kwast T. H., Eriksson, A., Willemsen, R., Weima, S. M., Hoogsteden, H. C., Hagemeijer, A. & Heldin, C.-H. (1991) Human malignant mesothelioma cell lines express PDGF β-receptors whereas cultured normal mesothelial cells express predominantly PDGF α-receptors, *Oncogene 6*, 2005–2011.

Wagner, J. C., Sleggs, C. A. & Marchand, P. (1960) Diffuse pleural mesothelioma and asbestos exposure in the North Western Cape Province, *Br. J. Ind. Med. 17*, 260–270.

Winkles, J. A. & Gay, C. G. (1991) Regulated expression of PDGF A-chain mRNA in human saphenous vein smooth muscle cells, *Biochem. Biophys. Res. Commun. 180*, 519–524.

Zenke, M., Kahn, P., Disela, C., Vennström, B., Leutz, A., Keegan, K., Hayman, M. J., Choi, H.-R., Yew, N., Engel, J. D. & Beug, H. (1988) *v-erbA* specifically suppresses transcription of the avian erythrocyte anion transporter (band 3) gene, *Cell 52*, 107–119.

CHAPTER 4.2

MECHANISM OF TRANSCRIPTIONAL ACTIVATION OF PDGF B-CHAIN EXPRESSION IN HUMAN MALIGNANT MESOTHELIOMA CELL LINES*

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ABSTRACT

Elevated PDGF B-chain mRNA expression was observed previously in human malignant mesothelioma cell lines as compared to cultured normal mesothelial cells. Because of its possible role as an autocrine growth factor in malignant mesothelioma cell lines, we investigated the mechanism behind this differential PDGF B-chain expression. We conclude that the difference in PDGF B-chain mRNA level between normal and malignant mesothelial cell lines is most probably determined at the transcriptional level, as in nuclear run off assays nuclear PDGF B-chain RNA could only be detected in malignant mesothelioma cells. Furthermore, the protein synthesis inhibitor cycloheximide did not affect the difference in PDGF B-chain mRNA levels between normal and malignant mesothelial cell lines.

Analysis of the PDGF B-chain promoter revealed that this region was hypersensitive for DNaseI and mediated basal activation in reporter CAT assays in both normal and malignant mesothelial cell lines. Further reporter gene analysis using a site-directed mutant construct strongly suggested that (part of) an activator is contained within the -64/-61 TCTC region. The *in vivo* footprint experiments indicated that actual binding of (a) factor(s) to this region and also to the region around the transcription start site only occurs in the PDGF B-chain-expressing malignant mesothelioma cell lines. This suggests that in normal mesothelial cells binding of such (a) factor(s) to the endogenous PDGF B-chain promoter is hindered by other mechanisms. Furthermore, several DNaseI hypersensitive (DH) sites were found in and around the PDGF B-chain transcription unit in both normal and malignant mesothelial cells. When tested in CAT assays, the region around DH -9.9, which was observed in both cell types, turned out to be an activator in malignant but not in normal mesothelial cells. However it did not function equally well in both orientations.

Taken together, a difference in transcription initiation seems to be the decisive factor for the elevated PDGF B-chain mRNA expression in malignant mesothelioma cells. Binding of one or more factors to the -64/-61 (TCTC) sequence of the promoter region seems to be relevant in this respect. Furthermore, an activator at -9.9 kb may contribute to the increased PDGF B-chain mRNA expression in the malignant mesothelioma cell lines as well.

* Submitted for publication.

INTRODUCTION

Platelet-derived growth factor (PDGF) is a connective tissue-cell mitogen that is composed of two disulfide-bonded polypeptides. Homodimeric (AA and BB) as well as heterodimeric (AB) forms of PDGF have been observed (1-3). The polypeptides are encoded by distinct genes, the PDGF A- and B-chain genes, which show high structural similarity (4). There are two structurally related but distinct PDGF receptors, denoted α and ß (5,6), which form homo- or heterodimers upon ligand binding (7,8). The PDGF receptors show different affinities for the dimeric PDGF isoforms. PDGF α-receptors bind all three dimeric forms of PDGF, whereas PDGF ß-receptors only bind PDGF-BB with high affinity (8-10).

Based on expression of PDGF chains and/or PDGF receptors in platelets, endothelial cells, smooth muscle cells and placental cytotrophoblasts, PDGF is thought to function in e.g. wound healing and developmental processes (reviewed in 11). Expression of PDGF (A-chain and/or B-chain) is also observed in various tumour tissues and cell lines (reviewed in 12,13). Co-expression of PDGF and one or both PDGF receptor subtypes is suggestive of autocrine (and paracrine) loops of growth stimulation in e.g. sarcoma and glioblastoma cells (14,15), and lung carcinomas (16). Direct evidence for autocrine stimulating activity of PDGF came from studies in which dominant-negative PDGF mutants and PDGF neutralizing antibodies were shown to revert the transformed phenotype of astrocytoma and glioblastoma cell lines (17,18).

Human malignant mesothelioma is a tumour of mesodermal origin which is most often found in the pleura. It is known that the incidence of malignant mesothelioma is strongly associated with asbestos exposure (19). In human malignant mesothelioma cell lines, but not in cultured normal mesothelial cells, PDGF B-chain mRNA expression was observed, whereas expression of PDGF A-chain mRNA was shown to be elevated in cultured malignant mesothelial cells as compared to their normal counterparts (20-22). Furthermore, most malignant mesothelioma cell lines were shown to express PDGF ß-receptor transcripts, while normal mesothelial cell lines predominantly expressed PDGF α-receptor mRNA (23). Immunofluorescence staining of normal and malignant mesothelial cells *in vitro* and *in vivo* with antibodies directed against PDGF and the PDGF receptors largely confirmed the mRNA expression pattern (24). The co-expression of PDGF B-chains and ß-receptors in malignant mesothelial cells *in vitro* and *in vivo*, is suggestive of autocrine growth stimulation (21,23,24).

Because of this postulated role for PDGF-BB as an autocrine growth factor, we studied the mechanism behind the elevated PDGF B-chain mRNA expression in malignant mesothelioma cell lines. We demonstrate by nuclear run off analysis that the increased PDGF B-chain mRNA level in malignant mesothelioma cell lines is most probably caused by elevated transcription. The promoter was found to be hypersensitive for DNaseI in both normal and malignant mesothelial cell lines. A DNaseI hypersensitive (DH) site is a nucleosome-free region in chromatin that is thought to be accessible to trans-acting factors (25). Recently, in other cell types proximal promoter elements of the PDGF B-chain gene (26-29) have been identified as well as several regions containing regulating elements outside the minimal promoter region (30-32). The promoter region was studied in more detail by *in vivo* footprinting and CAT reporter gene analysis to identify the promoter elements involved in regulation of PDGF B-chain transcription in malignant mesothelioma

cell lines. To find other regulating regions that are potentially involved in PDGF B-chain transcription, chromatin of normal and malignant mesothelial cell lines was further analyzed for the presence of DH sites in the PDGF B-chain transcription unit and its upstream and downstream flanking regions. In reporter assays these DH sites were tested for their transcription regulating potential in mesothelial cell lines.

MATERIALS AND METHODS

Cell lines and growth conditions

Experiments were performed using human malignant mesothelioma (Mero-25, Mero-41 and Mero-82) and normal mesothelial (NM-5, NM-20, NM-21) cell lines (24). All cell lines were routinely cultured in Ham's F10 medium (Gibco, Paisley, U.K.) with 15 % fetal calf serum (FCS). Epidermal growth factor (EGF; Collaborative Research Inc., Lexington, MA; 10 ng/ml) and hydrocortisone (HC; 0.4 µg/ml) were added to the culture medium of normal mesothelial cells. In some experiments cells were exposed to cycloheximide (CHX; Sigma, St. Louis, MO, USA; 10 µg/ml medium) for 2 h.

Probes

1.7 kb *Bam*HI PDGF B-chain (33) and 0.7 kb *Eco*RI-*Pst*I GAPDH (34) fragments were used as probes in Northern blot analysis. For nuclear run off analysis the 2.0 kb *Xho*I PDGF B-chain fragment from pSM1 (35) and the 1.25 kb *Pst*I ß-actin fragment from pAct (36) were both subcloned in pUC18 and subsequently spotted onto nitrocellulose filters prior to hybridization. For mapping of DH sites, subcloned sequences of human genomic PDGF B-chain clones were used (see also Figure 3). PR3 is the 0.61 kb *Sma*I-*Eco*RI fragment of pAO56, PR7 the 0.35 kb *Pst*I-*Hin*dIII fragment of pAO121, PR9 the 0.3 kb *Eco*RI-*Pst*I fragment of pAO68, PR12 the 0.9 kb *Hin*dIII-*Kpn*I fragment of pAO56, PR13 the 0.46 kb *Sma*I-*Hin*dIII fragment of pAO149, and PR16 the 0.38 kb *Pst*I-*Pvu*II fragment of pAO121. pAO56, pAO149, and pAO78 contain 11 and 5 kb *Eco*RI and 8 kb *Bam*HI fragments of cosmid clone ALLW-1283-Cl 21, respectively. Cosmid ALLW-1283-Cl 21, pAO68 and pAO121 were described elsewhere (37,38).

Reporter gene constructs for CAT analysis

pSV2CAT and pSuperCAT, a promoterless CAT construct, were used as positive and negative controls for CAT analysis, respectively. pSis-1758/+43CAT, pSis-425/+43CAT, and pSis-112/+43CAT have been described previously (31,39). pSis-425/+43CAT was used to construct unidirectional deletion mutants pSis-65/+43CAT, pSis-64/+43CAT, pSis-60/+43CAT, pSis-44/+43CAT, and pSis-36/+43CAT using exonuclease III (Promega, Madison, WI, USA) (39). pSis-112/+18mutaCAT is a site-directed mutant form of pSis-112/+18CAT, in which the sequence -64 TCTC -61 has been changed into -65 ATATC -61 (39). All these constructs were checked by sequencing. All other reporter constructs were made by cloning PDGF B-chain genomic fragments in sense (s) or antisense (a) orientation in the *Sma*I-site, upstream of the PDGF B-chain promoter in pSis1(s)CAT (=pSis-112/+43CAT) ([31,32] and Figure 4). PDGF B-chain-CAT fusion constructs 2(s), 3(s), and 4(s) contain the 170 bp (+302 to +472) *Hin*fI-*Xho*I, 297 bp (+472 to +761) *Xho*I-*Bam*HI, and 449 bp (+761 to +1210) *Bam*HI-*Bst*EII fragments of pAO121, respectively; 5(s) and 6(s) the 0.7 kb *Pst*I and 3.4 kb *Kpn*I fragments of pAO78, respectively; 8(s) the 3.2 kb *Eco*RI-*Xba*I fragment of pAO68; 10(s/a), 12(s/a), 13(s), and 14 (s/a) the 6 kb *Hin*dIII-*Bam*HI, 0.6 kb *Bst*UI, 0.7 kb *Sst*I-*Nar*I, and 1.5 kb *Pvu*II-*Bam*HI fragments of pAO56, respectively.

Northern and Southern blot analysis

DNA isolation and Southern blotting using Hybond-N membranes (Amersham Int., Amersham, UK) were performed according to standard procedures. RNA isolation and Northern blotting were performed as described elsewhere (22).

Nuclear run off assay

Isolation of nuclei, *in vitro* labeling of nuclear RNA and hybridization were described elsewhere (22).

Mapping of DNaseI hypersensitivity (DH) sites

Mapping of DH sites was performed largely according to the earlier described protocol (31). Nuclei were isolated from 6 x 10^8 normal or malignant mesothelial cells. After DNaseI and restriction enzyme digestion, electrophoresis, and blotting, DNA was hybridized overnight at 65°C in hybridization buffer containing 5 x SSPE, 0.1 % SDS, 0.1 % Ficoll, 0.1 % BSA, 0.1 % polyvinylpyrrolidone and 100 µg/ml salmon sperm DNA. Filters were washed at 65°C in 1 x SSPE, 0.1 % SDS. Autoradiography was performed at -80°C with intensifying screens using Kodak X-ray films.

In vivo footprinting

DMS treatment of subconfluent normal and malignant mesothelial cell cultures, DNA isolation and piperidine cleavage of *in vitro* or *in vivo* modified methylated guanine residues were all performed as described earlier (39).

Subsequently genomic footprinting was performed by means of ligation-mediated PCR (LM-PCR) (40) using PDGF B-chain promoter-specific primersets. PDGF B-chain primers 1s (5'-CATGGACTGAAGGGTTGCTC-3'; -190/-171), 2s (5'-CTCTCAGAGACCCCCTAAGCGCCCC-3'; -168/-144), and 3s (5'-AGACCCCCTAAGCG-CCCCGCCCTGG-3'; -161/-137) were used for lower strand analysis, whereas primers 1a (5'-CGCAAAGTATCTCTATCTAGGGAA-3'; +131/+108), 2a (5'-TAGGGAATGAAAAATGGGCGCTGGC3'; +114/+90), and 3a (5'-GGAATGAAAAATGGGCGCTGGCGGC-3'; +111/+86) were used for upper strand analysis. Firs strand synthesis (primers 1s and 1a), PCR amplification (primers 2s and 2a), and labeling reactions ([γ-^{32}P]ATP-labeled primers 3s and 3a) were all performed as described (39), using Vent DNA polymerase (*Thermococcus litoralis* DNA polymerase; New England Biolabs, Beverly, MA, USA). Mixtures were electrophoresed on 6% polyacrylamide sequencing gels, dried and subsequently exposed to Kodak X-ray films with intensifying screens at -80°C for 1 week.

Transfections and CAT assays

1.8-2.0 x 10^6 cells were seeded in 10 cm-dishes and grown in their standard culture medium for 24 h. Cells from subconfluent cultures were transfected with 1 µg pCH110 (ß-galactosidase expression vector; Pharmacia, Uppsala, Sweden) and 9 µg reporter construct in combination with 30 µg Lipofectin (Gibco BRL, Paisley, U.K.) in 3 ml serum-free OptiMEM culture medium (Gibco BRL) (41). For transfection two 10 cm-dishes with cells were used for each reporter construct. After 16 h 3 ml culture medium containing 30 % FCS (supplemented with EGF and HC for normal mesothelial cells) was added, resulting in a final concentration of 15 % FCS in the dishes. All cells were harvested 48 h later and cell lysates were prepared according to Sambrook et al. (42). The variation in transfection efficiency of the various reporter constructs was normalized by performing a ß-galactosidase assay. Amounts of protein corresponding to equal ß-galactosidase activity were used for CAT analysis, largely according to the protocol of Gorman et al. (43). After 1 h incubation at 37°C, 20 µl 4mM acetyl coenzyme A was readded and the incubation was continued for 1 h. The samples were extracted with ethyl acetate and analyzed on silica gel TLC plates (J.T. Baker, Phillipsburg, NJ). CAT activity was quantified using a PhosphorImager™ (Molecular Dynamics, Sunnyvale, CA, USA). In each experiment CAT activity of the promoterless pSuperCAT construct was subtracted from the values obtained for each construct. After correction CAT activities were determined relative to a reference construct. As CAT activities are thus expressed as percentage of reference activities, the mean and standard deviation were calculated on log-transformed values.

RESULTS

PDGF B-chain expression in mesothelioma cell lines is regulated at the transcriptional level

In order to study the PDGF B-chain nuclear RNA levels in normal and malignant mesothelial cell lines, nuclear run off assays were performed. No nuclear PDGF B-chain

RNA was observed in several normal mesothelial cell lines tested, whereas PDGF A-chain transcripts and transcripts of the constitutively transcribed ß-actin gene were clearly detectable (Figure 1 and data not shown). So the inability to detect B-chain transcripts in total RNA of normal mesothelial cell lines seems to be caused by absence of PDGF B-chain transcription rather than by messenger unstability. In human malignant mesothelioma cell lines nuclear PDGF B-chain transcripts could readily be observed, as shown for Mero-25 (Figure 1).

Culturing of normal mesothelial cells with the protein synthesis inhibitor cycloheximide (CHX) did not result in PDGF B-chain mRNA expression, as shown in Figure 2. CHX treatment did not cause any clear difference in expression of the 3.5 kb PDGF B-chain transcript in the malignant mesothelioma cell lines, not even when 20 or 30 µg/ml CHX was used. A smaller transcript that was seen in some of these cell lines was stabilized through CHX addition (Figure 2 and data not shown).

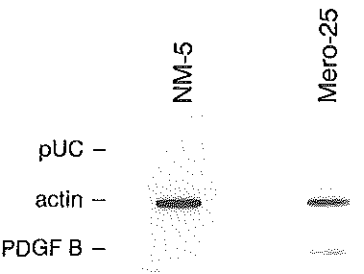

Figure 1. Nuclear run off analysis. ^{32}P-labeled nuclear RNA of normal mesothelial cell line NM-5 and malignant mesothelioma cell line Mero-25 was hybridized to nitrocellulose blots containing pUC, pUC + actin, and pUC + PDGF B-chain plasmids.

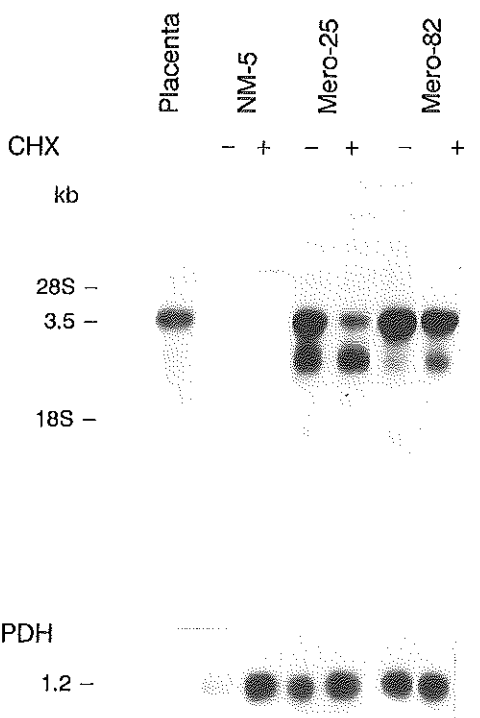

Figure 2. Northern blot analysis. 25 µg total RNA of cell lines NM-5, Mero-25, and Mero-82, cultured in the absence (-) or presence (+) of 10 µg/ml cycloheximide (CHX) for 2 h was blotted and hybridized to PDGF B-chain and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes.

Mapping of DH sites in the PDGF B-chain gene

Since these results pointed towards a difference in PDGF B-chain transcription between normal and malignant mesothelial cells, we studied the presence of DH sites in the PDGF B-chain transcription unit and its flanking regions in normal (NM-5) and malignant (Mero-25 and Mero-82) mesothelial cell lines; DNaseI hypersensitivity of a genomic DNA region is thought to correlate with accessibility of that region to trans-acting factors (25). Nuclei from NM-5, Mero-25 and Mero-82 were digested with DNaseI. After isolation, genomic DNA was digested with either *Eco*RI or *Hin*dIII. After hybridization with one of the probes indicated in Figure 3, DH sites were localized by calculating the length of the resulting

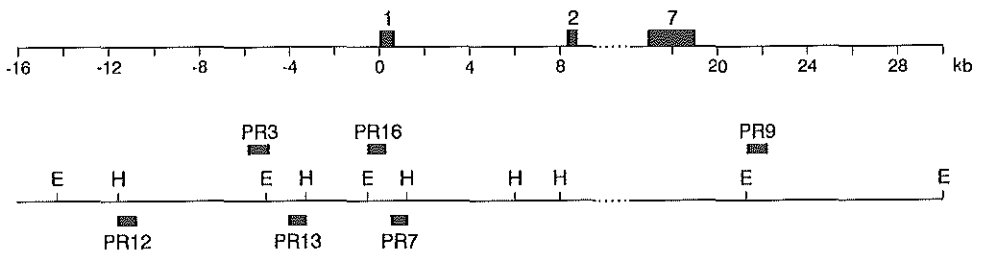

Figure 3. Schematic overview of restriction sites and probes used for mapping DH sites in the PDGF B-chain transcription unit. The numbered boxes in the upper panel indicate PDGF B-chain exons. Boxes in the lower panel indicate the genomic probes used for hybridization. E, *Eco*RI; H, *Hin*dIII.

fragments using a DNA marker. All DH sites that could be identified in these cell lines are summarized in Figures 4A and 4B.

Using probes PR16 and PR7 for analysis of the 22 kb *Eco*RI and 5.2 kb *Hin*dIII fragments, respectively, several DH sites were identified within the PDGF B-chain transcription unit. The promoter region was found to be DNaseI hypersensitive in Mero-25 and Mero-82 and also in NM-5. Several other DH sites (DH +0.4, DH +0.6, and DH +1.0) were observed in all cell lines as well. In contrast, two additional DH sites (DH +1.9 and DH +4.0) were found within the first intron of the PDGF B-chain gene of malignant mesothelioma cell lines only. There were no indications for DH sites in the other intron and exon sequences or in the region immediately upstream of the PDGF B-chain promoter in these cell lines.

DNA elements that are involved in regulation of transcription of a particular gene, can

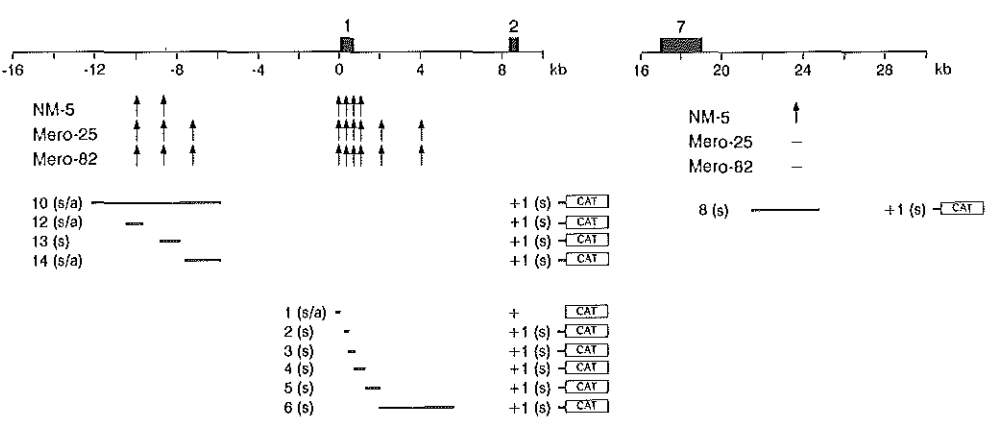

Figure 4. Localization of DH sites within (A), far upstream (A), and downstream (B) of the PDGF B-chain transcription unit and overview of CAT reporter constructs containing these DH sites. Normal mesothelial cell line NM-5 and malignant mesothelioma cell lines Mero-25 and Mero-82 were used for these studies. Numbered boxes designate PDGF B-chain exons. Arrows indicate location of identified DH sites. Fragment 1(s/a) corresponds to the -112/+43 B-chain promoter fragment. Bars indicate fragments cloned in sense (s) and/or antisense (a) orientation in front of the 1(s) fragment in CAT reporter gene constructs.

also be encountered in regions at a considerable distance of the transcription start site. We therefore examined the partly overlapping 9.2 kb *EcoR*I and 8.1 kb *Hin*dIII fragments upstream of the PDGF B-chain transcription unit with probes PR3 and PR13/PR12, respectively. Several DH sites (DH -9.9, DH -8.6 and DH -7.3) were observed in Mero-25 and Mero-82. In NM-5 only DH -9.9 and DH -8.6 were identified, whereas the region at -7.3 kb was hardly DNaseI hypersensitive. Finally, when using probe PR9 for the downstream region, (DH +23.7) was identified in NM-5, but not in Mero-25 and Mero-82.

Functional analysis of the DH promoter of the PDGF B-chain gene

Since the promoter region was found to be nucleosome-free in both normal and malignant mesothelial cell lines, we wanted to know whether in either of them factor(s) potentially involved in transcription, actually bind to sequences in this region. The PDGF B-chain proximal promoter region of both normal and malignant mesothelial cell lines was therefore studied in more detail by *in vivo* DMS footprint analysis. Guanine residue (G) -61

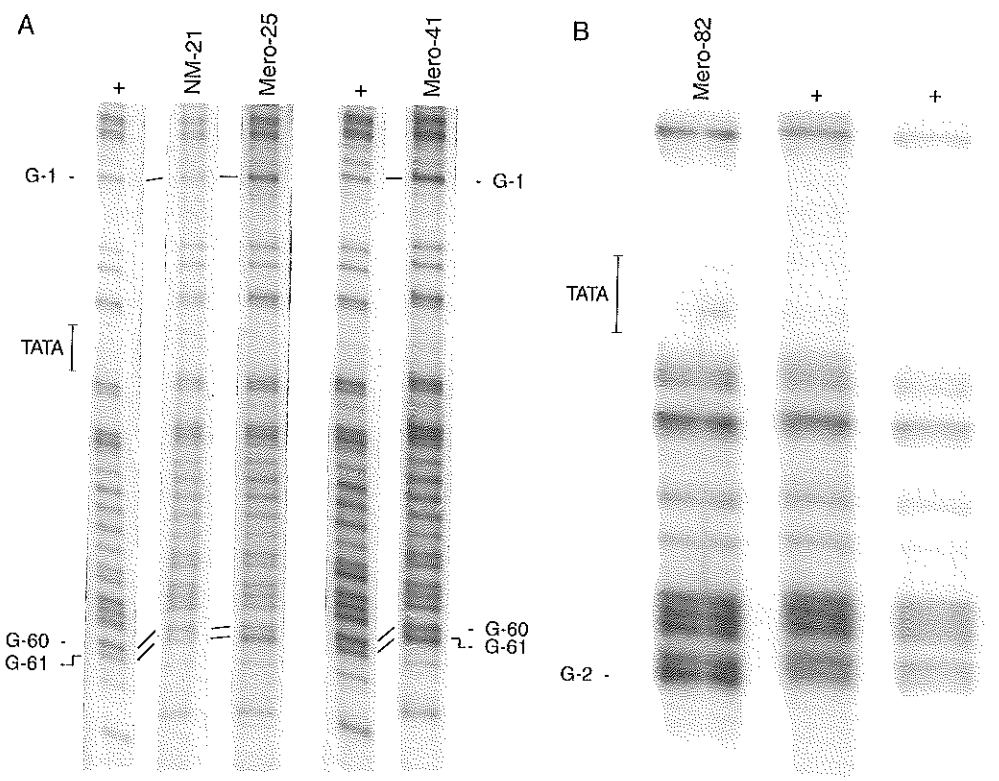

Figure 5. *In vivo* footprint analysis of the human PDGF B-chain promoter. Cells were treated with DMS *in vivo*, prior to piperidine cleavage and G residue sequencing. **A.** Numbers indicate positions of G residues in the lower strand of NM-21, Mero-25, and Mero-41 relative to the transcription initiation site. + *in vitro* treated naked DNA. **B.** Numbers indicate positions of G residues in the upper strand of Mero-82 relative to the transcription initiation site. + *in vitro* treated naked DNA.

in the lower strand was found to be hypermethylated in malignant mesothelioma cell lines as compared to the corresponding residue in *in vitro* DMS-treated naked DNA of mesothelial cells (Figure 5A). In normal mesothelial cells no such hypermethylated guanine residue was found at this position. We also detected hypermethylation at position G -1 in the lower strand in malignant mesothelioma cells, but not in normal mesothelial cells or in the *in vitro* DMS treated DNA samples (Figure 5A). Unfortunately the G -2 residue in the upper strand could be evaluated in only one of the mesothelioma DNA samples (Mero-82), showing hypermethylation as well, as compared to DMS-treated naked DNA (Figure 5B).

In an assay using chloramphenicol acetyl transferase (CAT) as reporter gene, the activity of the B-chain promoter region was tested in normal as well as malignant mesothelial cell lines. In both cases basal promoter activity was observed, using a fragment of 1758 bp upstream of the PDGF B-chain transcription start site. Most of the activity was retained when this fragment was narrowed down to the first 425, 112, or even 65 bp upstream of the transcription start (data not shown). When used in antisense direction the activity of the 112-bp fragment was much lower, indicating that the promoter needs a correct orientation for proper functioning. As we observed hypermethylation at G -61 in the *in vivo* footprint assay, we subsequently tested a series of PDGF B-chain promoter deletion mutants ranging from bp -64 to bp -36. A slight, though not statistically significant, decrease in activity was observed in the malignant mesothelioma cell lines between bp -64 and -60, as shown for Mero-82 (Figure 6; left part). As the effect was less clear in Mero-25 and Mero-41 (data not shown), the importance of the sequence between -64 and -60 was analyzed in more detail using a site-directed mutant. In this -112/+18mutaCAT construct the sequence -64 TCTC -61 was mutated to -65 ATATC -61 (40). In Mero-25, Mero-41, and Mero-82 a strong decrease in activity was observed upon mutation of the -64/-61

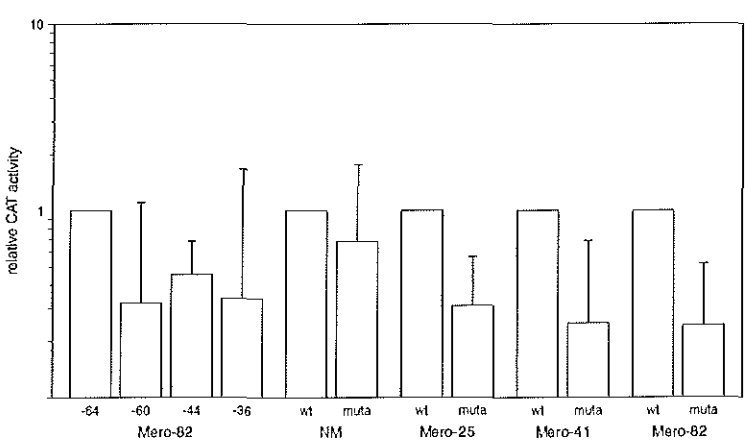

Figure 6. Relative CAT activities of cells transfected with various PDGF B-chain promoter/CAT fusion constructs. After normalization to ß-galactosidase activity from co-transfected expression vector pCH110, CAT activities were determined as percentage conversion. The CAT activity from the promoterless pSuperCAT construct was subtracted from each value. Corrected activities are indicated on a logarithmic scale relative to pSis-64/+43CAT (-64; left part) or pSis-112/+18CAT (wt; right part), which were defined as 1 in each cell line tested. All data are presented as means from 2-5 independent transfections. S.D. are indicated by errors bars.

sequence (Figure 6; right part). A similar but not significant effect was seen from this mutation in normal mesothelial cells (Figure 6; right part).

Functional analysis of other DH sites in the PDGF B-chain gene

All identified DH sites outside the promoter region were analyzed for their ability to regulate basal PDGF B-chain promoter-induced CAT activity in normal and malignant mesothelial cells. To this end reporter gene constructs with the sequences representing the DH sites cloned upstream of the 112 bp B-chain promoter [= construct 1(s)] (Figure 4), were transfected into these cells. No reproducible activity could be identified from sequences located in the first exon or first intron [constructs 2(s) to 6(s)], when tested in normal or malignant mesothelial cells (data not shown). A slight silencing effect of downstream fragment 8(s) was seen in normal mesothelial cells and not in Mero-82 (Figure 7).

Reporter constructs containing DH sites far upstream of the transcription unit were analyzed as well. In normal mesothelial cells construct 12 (containing DH -9.9) in either the sense (s) or antisense (a) orientation caused a small but not significant decrease in basal activity (Figure 7). In contrast, a clear 2 to 3-fold enhancing activity of the 12(a) construct was observed in Mero-25 and Mero-82 (Figure 7). When tested in the sense orientation the region around DH -9.9 [construct 12(s)] caused a strong increase in activity as well in Mero-82, but this strong enhancing effect was not seen in Mero-25 (Figure 7). To further elucidate the significance of these regions constructs 10(s) and 10(a), encompassing all identified DH sites in the -12/-6 kb region, were analyzed. A slight increase in basal PDGF

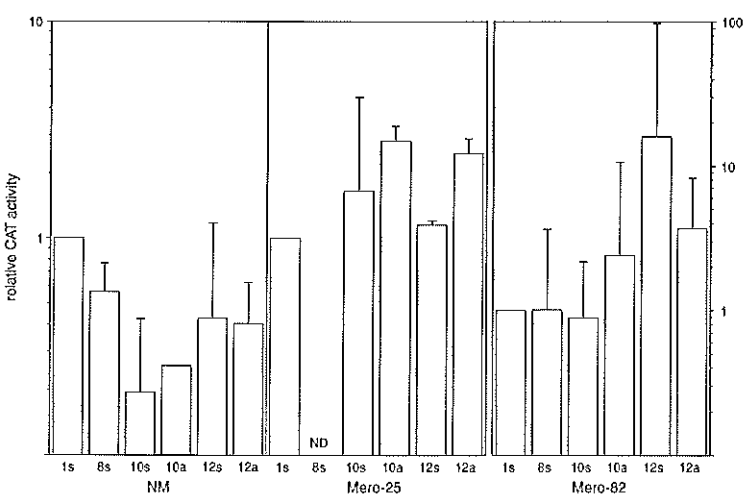

Figure 7. Relative CAT activities of cells transfected with various PDGF B-chain/CAT fusion constructs. After normalization to ß-galactosidase activity from co-transfected expression vector pCH110, CAT activities were determined as percentage conversion. The CAT activity from the promoterless pSuperCAT construct was subtracted from each value. Corrected activities are indicated on a logarithmic scale relative to pSis-112/+43 CAT [1(s)], which were defined as 1 in each cell line tested. Due to higher activities, a different scale is used for Mero-82 (right part). All data are presented as means from 2-4 independent transfections. S.D. are indicated by error bars. ND: not determined.

B-chain promoter activity was observed in Mero-82 using fragment 10(a), although this increase was lower than we had expected from the results with the 12(s/a) fragment (Figure 7). Fragment 10(s) did not show any enhancement at all. In Mero-25 constructs 10(s) and 10(a) demonstrated enhancing effects, similar to those observed with constructs 12(s) and 12(a) (Figure 7). Analysis of the entire -12/-6 kb region in normal mesothelial cells only revealed a silencing effect (Figure 7). Constructs 13(s) and 14(s/a), which encompass DH -8.6 and DH -7.3, respectively, did not seem to affect basal PDGF B-chain promoter activity in either the normal or the malignant cells in a reproducible way (data not shown).

DISCUSSION

In earlier studies PDGF B-chain mRNA expression was clearly detectable in human malignant but not in normal mesothelial cell lines (20,21). As there were no indications for consistent aberrations on chromosome 22 or for gene rearrangements or amplifications that could explain the elevated PDGF B-chain expression in mesothelioma cell lines (21), a transcriptional or post-transcriptional mechanism had to be considered. In this study we demonstrate by two different approaches, that the elevated steady-state PDGF B-chain mRNA level in malignant mesothelioma cell lines is caused by elevated transcription. By nuclear run off analysis no PDGF B-chain mRNA expression was observed in normal mesothelial cells, indicating a lack of transcription. Moreover, in normal mesothelial cells no PDGF B-chain mRNA could be detected after CHX treatment. In glioblastoma and bladder carcinoma cell lines and human umbilical vein endothelial cells (HUVEC) no stabilizing effect of CHX was seen either (44,45). In contrast, CHX clearly enhanced steady-state PDGF B-chain messenger levels in TPA-treated HL-60 cells and human monocytes (resting, LPS- and TPA-treated) (46,47). Fen and Daniel (48) showed that CHX treatment of microvascular endothelial cells specifically stabilized a smaller PDGF B-chain transcript, lacking the translation inhibiting 5' untranslated sequence (49). We found an increase in the expression level of a transcript of similar size after CHX treatment in a few of our cell lines. The PDGF B-chain mRNA half-life of 2 to 3 h, as determined in Actinomycin D-treated malignant mesothelioma cell lines (data not shown) is similar to reported B-chain half-lives in glioblastoma, bladder carcinoma, PC3, HeLa and TPA-treated K562 cells (31,44). Shorter half-lives were observed in HUVEC (44,45).

To further unravel activation of PDGF B-chain transcription in mesothelial cell lines, the promoter region was studied in more detail. Despite the clear difference in endogenous B-chain expression, the promoter region of the PDGF B-chain gene was found to be DNaseI hypersensitive in normal as well as malignant mesothelial cell lines. Moreover, the 1758 bp 5' flanking region of the PDGF B-chain gene was found to be able to drive transcription in both types of cell lines. Most of this activity was retained when narrowed down to -112 or -65 bp, relative to the transcription start identified by Van den Ouweland et al. (38). Further deletion of the promoter region resulted in a weak decrease in activity between -64 and -60. In addition, site-directed mutagenesis of the -64/-61 sequence caused a clear decreasing effect, suggesting that this region may harbour (part of) the binding sequence for (a) factor(s) involved in PDGF B-chain transcription. It is at present unknown why this

effect is not so clearly seen upon deletion of the region between -64 and -60, but it may be caused by an altered structural integrity in the deletion constructs, as compared to the site-directed mutant. A similar, but not significant, decreasing effect of the site-directed mutation was seen in normal mesothelial cells as well. This suggests that this factor is not exclusively present in malignant mesothelial cells. However, the footprint experiments show that actual binding of one or more factors to the region around G-61 and to the region around the transcription start site only seems to occur in PDGF B-chain-expressing malignant mesothelioma cell lines. In undifferentiated K562 cells and HeLa cells the -64/-61 region was found to be involved in activation of PDGF B-chain transcription as well, but the factor(s) binding at this TCTC sequence is (are) still unknown (39). Furthermore, a partly overlapping transcription activating region of the PDGF B-chain region -the SIS proximal element (SPE)- was observed in endothelial, osteosarcoma and K562 cells (27-29,39). This region appeared to contain the core binding site CCACCC (-61/-56) for the ubiquitously expressed transcription factor Sp1. In several cell types other regulatory elements were identified in the PDGF B-chain gene next to the SPE, especially in the region -100/-70 (26,28,29), but we could not find a consistent effect of these sequences on PDGF B-chain transcription in our mesothelioma cell lines. It remains to be determined which factor(s) play(s) a role in activation of PDGF B-chain transcription in malignant mesothelioma cell lines through binding to nucleotides around -61.

Collectively the results suggest that the absence of PDGF B-chain mRNA in normal mesothelial cell lines seems to be due to lack of B-chain transcript synthesis. Alternatively, a very low rate of PDGF B-chain transcription immediately followed by a very rapid degradation of the messenger may occur in these normal cells. The finding of a basal level of PDGF B-chain promoter-induced CAT expression in normal mesothelial cells may support the latter possibility, indicating that these cells in principle possess the necessary factors for PDGF B-chain transcription as well. A similar phenomenon is seen in human fibroblasts which do not show endogenous B-chain expression, but nevertheless demonstrate basal PDGF B-chain promoter-induced activity (26,31). Lack of clear nuclear RNA in normal mesothelial cells together with the data from the *in vivo* footprint experiment, suggest that in the context of the normal mesothelial cell genome the basal promoter activity is decreased or even totally repressed. Epigenetic mechanisms, such as methylation of promoter sequences or inaccessibility of the promoter due to nucleosome phasing, may play a role in this respect. However, preliminary Southern blot data of digests of *Msp*I and its methylation-sensitive isoschizomer *Hpa*II did not provide indications for differential methylation of the promoter region in normal and malignant mesothelial cell lines, but this should be studied in more detail. The possibility of nucleosome phasing, as suggested for human fibroblasts by Dirks et al. (31) appears less likely, since in both cell types the promoter region was found to be nucleosome-free. The precise mechanism of repression of B-chain mRNA transcription therefore remains to be determined in future studies.

In an attempt to identify other regulatory regions that may (partly) explain the observed difference in PDGF B-chain expression between normal and malignant mesothelial cells, we tested several of the identified DH sites for their transactivating potential. No enhancing activity was seen from first exon and first intron sequences. Moreover, the entire first intron region cloned upstream of its own promoter did not significantly affect promoter-induced reporter activity in mesothelioma cells (data not shown). In cervix

carcinoma and prostate carcinoma cell lines no activating influence from first exon or intron sequences was seen either (31). In contrast, Franklin et al. (30) showed enhancing effects of first intron sequences in choriocarcinoma and osteosarcoma cells, although the net effect of the entire first intron was not positive in the latter either. Since the DNA region contained in construct 8(s) was found to be DNaseI hypersensitive in normal mesothelial cells only, this weak silencer may contribute to the apparent lack of PDGF B-chain mRNA expression in normal mesothelial cells. In addition, constructs 12(s/a) conferred an even stronger downregulating effect to normal mesothelial cells. In Mero-25 and Mero-82 constructs 12(s/a) acted as an activator of transcription, suggesting that an enhancer is located around -9.9 kb. In sense orientation [12(s)] the effect was stronger in Mero-82 than in Mero-25. When tested in a larger construct, the enhancing effect of this -9.9 kb region was found to be weaker in both Mero-82 and Mero-25. The reason for this is unclear, as no consistent repressive (nor enhancing) effects were seen from constructs 13(s) and 14(s/a) containing identified DH sites at -8.6 and -7.3 kb, respectively. A comparable discrepancy was seen in cervix and prostate carcinoma cell lines, in which no repressing elements were found that could explain the difference in activation between constructs 10 and 12 (32).

In conclusion, in this paper we demonstrate that the difference in PDGF B-chain mRNA expression between normal and malignant mesothelial cells is mainly determined at the transcriptional level. In both normal and malignant mesothelial cell lines the PDGF B-chain promoter region was found to be nucleosome-free and to confer basal transcription activity when tested in reporter assays. However, *in vivo* footprint experiments demonstrate that actual binding (around bp -61) of one or more factors that seem to be involved in regulation of PDGF B-chain gene transcription only occurs in the PDGF B-chain-expressing mesothelioma cell lines. In addition, an enhancer-like element at the -9.9 kb region in malignant mesothelioma cells and silencer regions in normal mesothelial cells, seem to contribute to the elevated PDGF B-chain mRNA level in mesothelioma cell lines as well.

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REFERENCES

1. Stroobant, P. and M.D. Waterfield. 1984. Purification and properties of porcine platelet-derived growth factor. *EMBO J.* 3:2963-2967.
2. Heldin, C.-H., A. Johnsson, S. Wennergren, C. Wernstedt, C. Betsholtz, and B. Westermark. 1986. A human osteosarcoma cell line secretes a growth factor structurally related to a homodimer of PDGF A-chains. *Nature* 319:511-514.
3. Hammacher, A., U. Hellman, A. Johnsson, A. Östman, K. Gunnarson, B. Westermark, Å. Wasteson, and C.-H. Heldin. 1988. A major part of platelet-derived growth factor purified from human platelets is a heterodimer of one A and one B chain. *J. Biol. Chem.* 263:16493-16498.
4. Betsholtz, C., A. Johnsson, C.-H. Heldin, B. Westermark, P. Lind, M.S. Urdea, R. Eddy, T.B. Shows, K. Philpott, A.L. Mellor, T.J. Knott, and J. Scott. 1986. cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumor cell lines. *Nature* 320:695-699.
5. Hart, C.E., J.W. Forstrom, J.D. Kelly, R.A. Seifert, R.A. Smith, R. Ross, M. Murray, and D.F. Bowen-Pope. 1988. Two classes of PDGF receptors recognize different isoforms of PDGF. *Science* 240:1529-1531.

6. Heldin, C.-H., G. Bäckström, A. Östman, A. Hammacher, L. Rönnstrand, K. Rubin, M. Nistér, and B. Westermark. 1988. Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types. *EMBO J.* 7:1387-1393.
7. Heldin, C.-H., A. Ernlund, C. Rorsman, and L. Rönnstrand. 1989. Dimerization of B-type platelet-derived growth factor receptors occurs after ligand binding and is closely associated with receptor kinase activation. *J. Biol. Chem.* 264:8905-8912.
8. Seifert, R.A., C.E. Hart, P.E. Phillips, J.W. Forstrom, R. Ross, M.J. Murray, and D.F. Bowen-Pope. 1989. Two different subunits associate to create isoform-specific platelet-derived growth factor receptors. *J. Biol. Chem.* 264:8771-8778.
9. Claesson-Welsh, L., A. Eriksson, A. Morén, L. Severinsson, B. Ek, A. Östman, C. Betsholtz, and C.-H. Heldin. 1988. cDNA cloning and expression of human platelet-derived growth factor (PDGF) receptor specific for B-chain-containing PDGF molecules. *Mol. Cell. Biol.* 8:3476-3486.
10. Claesson-Welsh, L., A. Eriksson, B. Westermark, and C.-H. Heldin. 1989. cDNA cloning and expression of the human A type PDGF receptor establishes structural similarity to the B type receptor. *Proc. Natl. Acad. Sci. USA* 86:4917-4921.
11. Ross, R., D.F. Bowen-Pope, and E.W. Raines. 1990. Platelet-derived growth factor and its role in health and disease. *Phil. Trans. R. Soc. Lond.* 327:155-169.
12. Raines, E.W., D.F. Bowen-Pope, and R. Ross. 1990. Peptide Growth Factors and their receptors. *In* Handbook in Experimental Pharmacology. M.B. Sporn and A.B. Roberts, editors. Springer Verlag, Heidelberg. Vol. 95, part 1, 173-262.
13. Westermark, B. and C.-H. Heldin. 1991. Platelet-derived growth factor in autocrine transformation. *Cancer Res.* 51:5087-5092.
14. Leveen, P., L. Claesson-Welsh, C.-H. Heldin, B. Westermark, and C. Betsholtz. 1990. Expression of messenger RNAs for platelet-derived growth factor and its receptors in human sarcoma cell lines. *Int. J. Cancer* 46:1066-1070.
15. Nistér, M., T. Libermann, C. Betsholtz, M. Pettersson, L. Claesson-Welsh, C.-H. Heldin, J. Schlessinger, and B. Westermark. 1988. Expression of messenger RNA for platelet-derived growth factor and transforming growth factor-α and their receptors in human malignant glioma cell lines. *Cancer Res.* 48:3910-3918.
16. Antoniades, H.N., T. Galanopoulos, J. Neville-Golden, and C.J. O'Hara. 1992. Malignant epithelial cells in primary human lung carcinomas coexpress in vivo platelet-derived growth factor (PDGF) and PDGF receptor mRNA and their protein products. *Proc. Natl. Acad. Sci. USA* 89:3942-3946.
17. Shamah, S.M., C.D. Stiles, and A. Guha. 1993. Dominant-negative mutants of platelet-derived growth factor revert the transformed phenotype of human astrocytoma cells. *Mol. Cell. Biol.* 13:7203-7212.
18. Vassbotn, F., A. Östman, N. Langeland, H. Holmsen, B. Westermark, C.-H. Heldin, and M. Nistér. 1994. Activated platelet-derived growth factor autocrine pathway drives the transformed phenotype of a human glioblastoma cell line. *J. Cell. Physiol.* 158:381-389.
19. Wagner, J.C., C.A. Sleggs, and P. Marchand. 1960. Diffuse pleural mesothelioma and asbestos exposure in the North Western Cape Province. *Br. J. Ind. Med.* 17:260-271.
20. Gerwin, B.I., J.F. Lechner, R.R. Reddel, A.B. Roberts, K.C. Robbins, E.W. Gabrielson, and C.C. Harris. 1987. Comparison of production of transforming growth factor-beta and platelet-derived growth factor by normal humam mesothelial cells and mesothelioma cell lines. *Cancer Research* 47:6180-6184.
21. Versnel, M.A., A. Hagemeijer, M.J. Bouts, Th.H. Van der Kwast, and H.C. Hoogsteden. 1988. Expression of c-sis (PDGF B-chain) and PDGF A-chain genes in ten human malignant mesothelioma cell lines derived from primary and metastatic tumors. *Oncogene* 2:601-605.
22. Langerak, A.W., R.P.H. Dirks, and M.A. Versnel. 1992. Splicing of the platelet-derived growth factor A-chain mRNA in human malignant mesothelioma cell lines and regulation of its expression. *Eur. J. Biochem.* 208:589-596.
23. Versnel, M.A., L. Claesson-Welsh, A. Hammacher, M.J. Bouts, Th.H. Van der Kwast, A. Eriksson, R. Willemsen, S.M. Weima, H.C. Hoogsteden, A. Hagemeijer, and C.-H. Heldin. 1991. Human malignant mesothelioma cell lines express PDGF β-receptors whereas cultured normal mesothelial cells express predominantly PDGF α-receptors. *Oncogene* 6:2005-2011.
24. Langerak, A.W., P.A.J.M. De Laat, C.A.J. Van der Linden-van Beurden, M. Delahaye, Th.H. Van der Kwast, H.C. Hoogsteden, R. Benner, and M.A. Versnel. 1995. Expression of PDGF and PDGF receptors in human malignant mesothelioma *in vitro* and *in vivo*. *J. Pathol.* In press.
25. Gross, D.S. and W.T. Garrard. 1988. Nuclease hypersensitive sites in chromatin. *Ann. Rev. Biochem.* 57:159-197.
26. Pech, M., C.D. Rao, K.C. Robbins, and S.A. Aaronson. 1989. Functional identification of regulatory elements within the promoter region of platelet-derived growth factor 2. *Mol. Cell. Biol.* 9:396-405.
27. Jin, H.M., M.L. Brady, and W.E. Fahl. 1993. Identification and characterization of an essential, activating regulatory element of the human *SIS/PDGF-B* promoter in human megakaryocytes. *Proc. Natl. Acad. Sci. USA* 90:7563-7567.

28. Jin, H.M., D.F. Robinson, Y. Liang, and W.E. Fahl. 1994. *SIS/PDGF-B* promoter isolation and charcterization of regulatory elements necessary for basal expression of the *SIS/PDGF-B* gene in U2-OS osteosarcoma cells. *J. Biol. Chem.* 269:28648-28654.
29. Khachigian, L.M., J.W.U. Fries, M.W. Benz, D.T. Bonthron, and T. Collins. 1994. Novel *cis*-acting elements in the human platelet-derived growth factor B-chain core promoter that mediate gene expression in cultured vascular endothelial cells. *J. Biol. Chem.* 269:22647-22656.
30. Franklin, G.C., M. Donovan, G.I.R. Adam, L. Holmgren, S. Pfeifer-Ohlsson, and R. Ohlsson. 1991. Expression of the human PDGF-B gene is regulated by both positively and negatively acting cell type-specific regulatory elements located in the first intron. *EMBO J.* 10:1365-1373.
31. Dirks, R.P.H., H.J. Jansen, J. Gerritsma, C. Onnekink, and H.P.J. Bloemers. 1993. Localization and functional analysis of DNaseI-hypersensitive sites in the human *c-sis/PDGF-B* gene transcription unit and its flanking regions. *Eur. J. Biochem.* 211:509-519.
32. Dirks, R.P.H., H.J. Jansen, C. Onnekink, R.J.A. de Jonge, and H.P.J. Bloemers. 1993. DNaseI-hypersensitive sites located far upstream of the human c-*sis*/PDGF B gene comap with transcriptional enhancers and a silencer and are preceded by (part of) a new transcription unit. *Eur. J. Biochem.* 216:487-495.
33. Groffen, J., N. Heisterkamp, J.R. Stephenson, A. Geurts van Kesssel, A. De Klein, G. Grosveld, and D. Bootsma. 1983. C-sis is translocated from chromosome 22 to chromosome 9 in chronic myeolocytic leukemia. *J. Exp. Med.* 158:9-15.
34. Benham, F.J., S. Hodgkinson, and K.E. Davies. 1984. A glyceraldehyde-3-phosphate dehydrogenase pseudogene on the short arm of the human X-chromosomes defines a multigene family. *EMBO J.* 3:2635-2640.
35. Josephs, S.F., L. Ratner, M.F. Clarke, E.H. Westin, M.S. Reitz, and F. Wong-Staal. 1984. Transforming potential of human c-sis nucleotide sequences encoding platelet-derived growth factor. *Science* 225:636-639.
36. Dodemont, H.J., P. Soriano, W.J. Quax, F. Ramaekers, J.A. Lenstra, M.A. Groenen, G. Bernardi, and H. Bloemendal. 1982. The genes coding for the cytoskeletal proteins actin and vimentin in warmblooded vertebrates. *EMBO J.* 1:167-171.
37. Van den Ouweland, A.W.M., M.L. Breuer, P.H. Steenbergh, J.A. Schalken, H.P.J. Bloemers, and W.J.M. Van der Ven. 1985. Comparative analysis of the human and feline c-sis proto-oncogenes. Identification of 5' human c-sis coding sequences that are not homologous to the transforming gene of Simian Sarcoma Virus. *Bioch. Biophys. Acta* 825:140-147.
38. Van den Ouweland, A.W.M., A.J.M. Roebroek, J.A. Schalken, C.A.A. Claessen, H.P.J. Bloemers, and W.J.M. Van der Ven. 1986. Structure and nucleotide sequence of the 5' region of the human and feline c-sis protooncogenes. *Nucl. Acids Res.* 14:765-778.
39. Dirks, R.P.H, H.J. Jansen, H.J. Van Gerven, C. Onnekink, and H.P.J. Bloemers. 1995. *In vivo* footprinting and functional analysis of the human c-*sis*/PDGF B gene promoter provides evidence for two binding sites for transcriptional activators. *Nucl. Acids Res.* 23:1119-1126.
40. Garrity, P.A. and B.J. Wold. 1992. Effects of different DNA polymerases in ligation-mediated PCR: enhanced genomic sequencing and *in vivo* footprinting. *Proc. Natl. Acad. Sci. USA* 89:1021-1025.
41. Felgner, P.L., T.R. Gadek, M. Holm, R. Roman, H.W. Chan, M. Wenz, J.P. Northrop, G.M. Ringold, and M. Danielsen. 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA* 84:7413-7417.
42. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning. A Laboratory Manual (2nd ed.) Cold Spring Harbor Press, Cold Spring Harbor, NY, USA.
43. Gorman, C.M., L.F. Moffat, and B.H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2:1044-1051.
44. Press, R.D., D. Samols, and D.A. Goldthwait. 1988. Expression and stability of c-sis mRNA in human glioblastoma cells. *Biochem.* 27:5736-5741.
45. Gay, C.G. and J.A. Winkles. 1991. The half-lives of platelet-derived growth factor A- and B-chain mRNAs are similar in endothelial cells and unaffected by heparin-binding growth factor-1 or cycloheximide. *J. Cell. Physiol.* 147:121-127.
46. Sariban, E. and D. Kufe. 1988. Expression of the platelet-derived growth factor 1 and 2 genes in human myeloid cell lines and monocytes. *Cancer Res.* 48:4498-4502.
47. Nagaoka, I., A. Someya, K. Iwabuchi, and T. Yamashita. 1991. Comparative studies on the platelet-derived growth factor-A and -B gene expression in human monocytes. *Comp. Biochem. Physiol.* 2:313-319.
48. Fen, Z. and T.O. Daniel. 1991. 5' Untranslated sequences determine degradative pathway for alternate PDGF B/c-sis mRNA's. *Oncogene* 6:953-959.
49. Rao, C.D., M. Pech, K.C. Robbins, and S.A. Aaronson. 1988. The 5' untranslated sequence of the *c-sis*/platelet-derived growth factor 2 transcript is a potent translational inhibitor. *Mol. Cell. Biol.* 8:284-292.

CHAPTER 4.3

REGULATION OF DIFFERENTIAL EXPRESSION OF PDGF α- AND ß-RECEPTOR mRNA IN NORMAL AND MALIGNANT HUMAN MESOTHELIAL CELL LINES*

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ABSTRACT

In earlier studies we showed that the expression patterns of platelet-derived growth factor (PDGF) α- and ß-receptors differ between normal and malignant mesothelial cell lines. Normal mesothelial cells predominantly express PDGF α-receptor mRNA and protein, whereas most malignant mesothelioma cell lines produce PDGF ß-receptor mRNA and protein. In this paper we studied regulation of this differential PDGF receptor mRNA expression. Such an analysis is of importance in view of the suggested PDGF autocrine activity involving the PDGF ß-receptor in mesothelioma cells.

The results obtained in this study demonstrate that malignant mesothelioma cell lines are not only capable of PDGF ß-receptor transcription but of α-receptor transcription as well, as evidenced from run off analysis and RT-PCR using α-receptor specific primers. However, the fact that PDGF α-receptor mRNA could not be detected by Northern blot analysis, even after cycloheximide treatment, suggests a difference in steady-state PDGF α-receptor mRNA expression levels between normal and malignant mesothelial cell lines, which is likely to be caused by a post-transcriptional mechanism. In normal mesothelial cells a half-life of more than 6 h was observed for PDGF α-receptor mRNA. In the majority of malignant mesothelioma cell lines clear PDGF ß-receptor mRNA expression was seen. The half-life of the PDGF ß-receptor transcript was at least 6 h in these cells. In contrast, no PDGF ß-receptor transcription was observed in run off assays in normal mesothelial cells, suggesting that differences in ß-receptor transcriptional initiation most probably account for the inability to clearly detect PDGF ß-receptor transcripts in these cells.

Stimulation with transforming growth factor-ß1 (TGF-ß1) revealed decreased PDGF α-receptor mRNA expression in normal mesothelial cells, whereas the effect on PDGF ß-receptor mRNA in the malignant mesothelioma cell lines was variable. Although this effect of TGF-ß1 stimulation cannot entirely explain the differential PDGF receptor expression pattern, TGF-ß1 may nevertheless play a role in downregulation of an (already) low PDGF α-receptor mRNA level in malignant mesothelioma cell lines.

Post-transcriptional and transcriptional mechanisms most probably account for the

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observed differences in expression of PDGF α- and ß-receptor mRNA, respectively, in normal and malignant mesothelial cell lines. This differential regulation underscores the relevance of mesothelial cell lines as a model system for future studies on regulation of PDGF receptor expression.

INTRODUCTION

Platelet-derived growth factor (PDGF) is a mitogenic factor for cells of mesenchymal origin (14), that is composed of two disulfide-bonded polypeptides. These polypeptide chains are encoded by the distinct, but structurally related, PDGF A-chain and B-chain genes (5). Homodimeric (AA and BB) as well as heterodimeric (AB) forms of PDGF were observed. In addition, two homologous PDGF receptor subtypes have been described, the PDGF α-receptor and PDGF ß-receptor (13). These two receptors show different affinities for the various dimeric PDGF forms. PDGF α-receptors bind all PDGF isoforms (AA, AB, and BB) with high affinity, whereas PDGF ß-receptors only show high affinity binding of PDGF-BB (25). Ligand binding results in receptor dimerization and subsequent activation through cross-phosphorylation on tyrosine residues (17).

PDGF and PDGF receptors, expressed by various cell types like platelets, macrophages and cytotrophoblasts (for a review, see 24) play a role in e.g. wound healing and developmental processes. PDGF has been suggested to play a role in several tumour types as well (for a review, see 23,33). Evidence for autocrine activity of PDGF has been presented in glioblastoma and astrocytoma cell lines (26,27).

Human malignant mesothelioma is a tumour of mesodermally-derived tissues that is predominantly observed in the pleura. Based on *in vitro* and *in vivo* expression data, PDGF was suggested to play a role in the tumorigenesis of malignant mesothelioma (19,28,30). In summary, malignant mesothelioma cells were found to express PDGF A-chain and B-chain mRNA at high levels, whereas normal mesothelial cells only expressed low levels of PDGF A-chain mRNA. Furthermore, expression of PDGF α-receptor mRNA and protein was demonstrated in non-malignant mesothelial cells, whereas in mesothelioma cells PDGF ß-receptor transcripts and proteins were observed in most cases. Membrane-bound PDGF α-receptor proteins were also found to be expressed by the latter (19).

In view of the involvement of PDGF and PDGF receptors in several physiological and pathophysiological processes, transcriptional regulation of PDGF chains and receptors is an important topic to study. Transcriptional regulation constitutes the first regulatory level of the biological action of PDGF as it determines the availability of ligands and signal-transducing receptors. In our normal and malignant mesothelial cell lines we previously analyzed regulation of PDGF A-chain and B-chain transcription (18 and unpublished work). We now report on the regulation of the differential PDGF receptor mRNA expression in normal and malignant mesothelial cell lines, as insight into regulation of PDGF receptors in mesothelioma cells is relevant in view of the possible PDGF-driven autocrine loop in these cells. It has been shown in several studies that mRNA expression of PDGF chains and PDGF receptors is regulated by e.g. acidic fibroblast growth factor and interleukin-1 (22,34,35). Transforming growth factor-ß (TGF-ß) is probably one of the most extensively studied regulators of PDGF receptor expression. TGF-ß stimulation has been shown to result in

downregulation of PDGF α-receptor mRNA in e.g. fibroblasts and smooth muscle cells (3,21), whereas upregulation of PDGF ß-receptor messenger levels was seen in smooth muscle and mesangial cells (12,15). As TGF-ß has been shown to be endogenously produced by mesothelial and mesothelioma cells (9,11,20), we evaluated whether TGF-ß may contribute to the differential expression of PDGF α- and ß-receptors in mesothelial cell lines. Although studies on regulatory elements involved in transcription of the PDGF A-chain and B-chain genes have been performed in many cell types (for a recent review, see (16)), similar reports on PDGF receptor elements have been limited so far. Recently, studies on the promoter and upstream regulatory elements of the murine (32) and human (1) PDGF α-receptor have been published. Their differential expression pattern makes the mesothelial cell lines important as model system for studies on PDGF receptor regulation. The results discussed in this study are therefore also relevant for the general understanding of regulation of PDGF α- and ß-receptor expression.

MATERIALS AND METHODS

Cell lines and growth conditions

Experiments were performed using the human malignant mesothelioma cell lines Mero-14, Mero-25, Mero-41, Mero-48c, Mero-72, Mero-82, Mero-84, and Mero-95 and the normal human mesothelial cell lines NM-9, NM-12, NM-21, NM-23, and NM-25 (19,29). The cell lines were routinely cultured in Ham's F10 medium (Gibco, Paisley, U.K.) with 15 % fetal calf serum (29). Epidermal growth factor (EGF; Collaborative Research Inc., Lexington, MA, USA; 10 ng/ml) and hydrocortisone (HC; 0.4 µg/ml) were added to the culture medium of normal mesothelial cells. In some experiments cells were exposed to cycloheximide (CHX; Sigma, St. Louis, MO, USA) at a concentration of 10 µg/ml medium for 2 h or to actinomycin D (Act D; Merck Sharp & Dohme Int., Rahway, NJ, USA) at a concentration of 5 µg/ml medium for 1-6 h. Prior to TGF-ß1 treatment, cells were cultured under serum-free conditions in Ham's F10 medium (+EGF/HC for normal cells) supplemented with 0.1 mg/ml BSA (Sigma), 10 µg/ml human transferrin (Behringwerke AG, Marburg, Germany), 10 µg/ml insulin (Sigma), and 50 nM sodium selenite (Merck, Darmstadt, Germany) for 24 h. Subsequent stimulation with human TGF-ß1 (2 ng/ml; 24 h; R&D, Abingdon, U.K.) was performed in this serum-free medium as well.

Probes

For hybridization of Northern blots the 1.5 kb *Eco*RI (extracellular) PDGF α-receptor (7), the 1.7 kb *Eco*RI-*Hin*dIII (extracellular) PDGF ß-receptor (6), and the 0.7 kb *Eco*RI-*Pst*I GAPDH (4) cDNA fragments were used as probes. For nuclear run off analysis the same extracellular PDGF α-receptor and ß-receptor fragments (6,7) as well as the 1.25 kb *Pst*I ß-actin fragment from pAct (8) were all subcloned in the multicloning site of pUC and subsequently spotted onto nitrocellulose filters prior to hybridization.

Northern and Southern blot analyses

RNA isolation, Northern blotting and subsequent hybridization were performed as described elsewhere (18). Isolation of chromosomal DNA and transfer to Hybond-N (Amersham, UK) membranes were performed according to standard procedures. Hybridization and autoradiography were described previously (28).

Nuclear run off assay

Isolation of nuclei, *in vitro* labeling of nuclear RNA and hybridization were described elsewhere (18).

RT-PCR analysis

For reverse transcription (RT) 1 µg total RNA was denatured at 65°C for 10 min and mixed with 10 µg/ml oligo$(dT)_{15}$ (Pharmacia, Uppsala, Sweden), 2.5 OD_{260}/ml $(dN)_6$ (Pharmacia), avian myoblastoma virus (AMV) RT buffer (50 mM Tris-HCl, pH 8.3; 50 mM KCl; 10 mM $MgCl_2$; 1 mM dithiothreitol; 1 mM EDTA;

10 µg/ml bovine serum albumin), 1mM dNTPs, 1mM spermidine-HCl (Sigma), 40 U RNAsin (Promega, Madison, WI, USA), and 5 U AMV RTase (Promega) and incubated for 55 min at 39°C. For amplification of PDGF α-receptor sequences cDNA was mixed with *Taq* buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% (m/v) gelatin), 0.2 mM dNTPs, 20 pmol sense primer αR-7 (5'-CTGGAAGAAAT-CAAAGTCCCATCC-3';bp 909-932 according to the sequence in (7)), 20 pmol antisense primer αR-8 (5'-TGAGCCATGGTGATCATCGACC-3'; bp 1388-1409), and 1 U *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA). Fourty cycles of 30 sec at 94°C, 30 sec at 65°C, and 30 sec at 72°C were performed. The PDGF α-receptor cDNA clone pSV7d15EB and a full-length PDGF ß-receptor cDNA clone were used as positive and negative controls, respectively (6,7). No amplified products were seen using these primers on chromosomal DNA and non-reverse transcribed RNA. All samples were checked for the amount of analyzable cDNA using specific HPRT gene primers (fourty cycles of 30 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C). Ethidium bromide analysis and hybridization of filters with amplified PCR products were performed as described previously (18). PDGF α-receptor-specific oligo αR-3A (5'-TCACTGAGATCACCACTGATGTGG-3'; bp 1207-1230) was used as probe for hybridization.

RESULTS

Differential regulation of PDGF α- and ß-receptor mRNA expression in mesothelial cell lines

As normal and malignant mesothelial cell lines display a differential PDGF receptor mRNA expression pattern and as there are no indications for consistent cytogenetic and/or genomic aberrations, run off assays were performed to study the nuclear PDGF α- and ß-receptor mRNA levels in these cell lines. Nuclear PDGF α-receptor RNA could be demonstrated in both normal and malignant mesothelial cell lines (Table 1). In Figure 1 this is shown for NM-9 and Mero-14 as representative examples. Nuclear PDGF ß-receptor transcripts were only detectable in the malignant mesothelioma cell lines and hardly or not in the normal mesothelial cell lines tested (Figure 1 and Table 1). All cell lines tested clearly expressed nuclear RNA of the constitutively transcribed ß-actin gene.

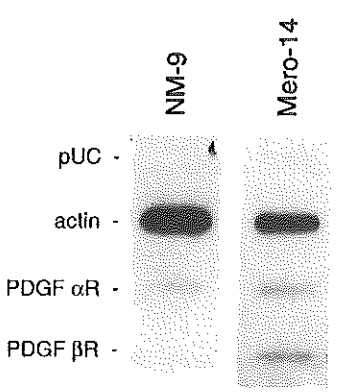

Figure 1. Nuclear run off analysis using ^{32}P-labeled nuclear RNA of normal mesothelial (NM-9) and malignant mesothelioma (Mero-14) cell lines. The nitrocellulose blots contained plasmids pUC, pUC plus actin, pUC plus PDGF α-receptor, and pUC plus PDGF ß-receptor.

Culturing of several normal and malignant mesothelial cells with the protein synthesis inhibitor cycloheximide (CHX; 10 µg/ml) did not significantly affect the PDGF ß-receptor mRNA levels in both cell types (Table 1). This is shown for NM-9 and Mero-14 in Figure 2. PDGF ß-receptor mRNA expression was hardly or not detectable in untreated as well as CHX-treated normal mesothelial cells. Furthermore, CHX treatment did not alter the differential expression of PDGF α-receptor transcripts in normal and malignant mesothelial cell lines, as determined by Northern blot analysis (Figure 2 and Table 1). In CHX-treated malignant mesothelioma cell lines no PDGF α-receptor mRNA could be demonstrated. Moreover, the use of higher concentrations of CHX (20-30 µg/ml) still did not result in detectable PDGF α-receptor expression in these cells (data not shown).

TABLE 1. Summary of data on PDGF α-receptor and ß-receptor mRNA detection in normal and malignant mesothelial cell lines under various experimental conditions.

cell line	PDGF α-receptor				PDGF ß-receptor		
	nuclear RNA (run off assay)	mRNA (CHX treated samples)	mRNA (RT-PCR)	mRNA half-life (Act D treated samples)	nuclear RNA (run off assay)	mRNA level (CHX treated samples)	mRNA half-life (Act D treated samples)
NM-9	+	+	+	min. 4-6 h	-	-	no mRNA detectable
NM-12	+	+	ND	ND	-	-	ND
NM-23	ND	ND	+	ND	ND	ND	ND
Mero-14	+	-	+/-	no mRNA detectable	+	+	min. 4-6 h
Mero-25	+	-	+/-	ND	+/-	+/-	ND
Mero-41	ND	-	+/-	ND	+	+	ND
Mero-48c	ND	-	+/-	ND	+	+	ND
Mero-82	+	-	+/-	no mRNA detectable	+/-	+/-	min. 4-6 h
Mero-84	+	ND	ND	ND	+	ND	ND

ND: not determined.

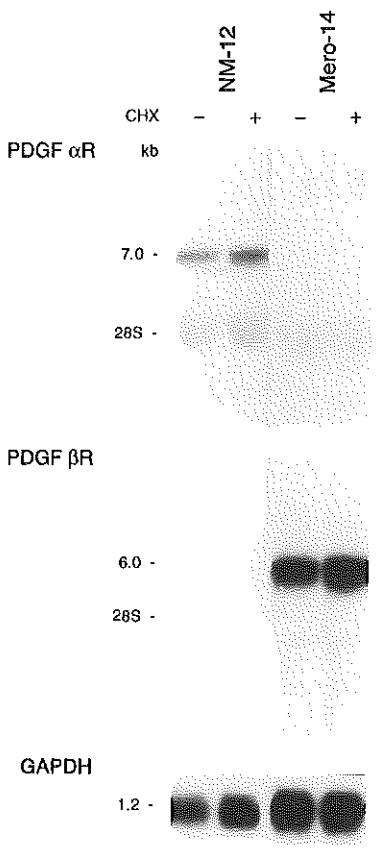

Figure 2. Northern blot analysis of total RNA from normal mesothelial (NM-9) and malignant mesothelioma (Mero-14) cell lines. Total RNA (25 µg) of cells cultured in the absence (-) or presence (+) of 10 µg/ml cycloheximide (CHX) was blotted and hybridized to PDGF α-receptor, PDGF ß-receptor, and GAPDH probes.

Detection of PDGF α-receptor mRNA by RT-PCR

To obtain further evidence for PDGF α-receptor gene expression in malignant mesothelioma cell lines, we performed RT-PCR analysis. Amplification of PDGF α-receptor cDNA clone pSV7d15EB (used as positive control) with primers αR-7 and αR-8 resulted in a 501 bp band. A fragment of similar size could also be amplified from cDNA of normal mesothelial cells (Figure 3 and Table 1). In the majority of our malignant mesothelioma cell lines such a 501 bp band could be detected as well, although the intensity was generally much lower than in normal mesothelial cell lines (Figure 3 and Table 1). However, distinct samples of a single malignant mesothelioma cell line were not always positive, further indicating low expression levels indeed. Since the PDGF α- and ß-receptor sequences are quite homologous in many regions, cross-reactivity of the αR-7 and αR-8 primers with ß-receptor sequences in these cells had to be excluded. Therefore, we checked the specificity of the PDGF α-receptor primers in various ways. *Eco*RV digestion of the amplified fragments resulted in the expected 244 and 257 bp PDGF α-receptor-cDNA fragments (data not shown), whereas hybridization with the αR-3A oligo further confirmed the PDGF α-receptor origin of the amplified cDNA fragments (Figure 3). Furthermore, the lack of amplification of the PDGF ß-receptor cDNA construct with these primers excluded cross-reactivity with PDGF ß-receptor sequences. Finally, genomic DNA, non-reverse transcribed RNA, and H_2O controls were all negative. Despite the low expression levels, the results are in agreement with the data obtained in run off assays, indicating that malignant mesothelioma cells are in principle capable of producing PDGF α-receptor mRNA as well.

Stability of PDGF α- and ß-receptor transcripts

The stability of the PDGF α- and ß-receptor transcripts was studied by Northern blot analysis in a few normal and malignant mesothelial cell lines (NM-9, Mero-14, Mero-82), which were treated with actinomycin D (Act D) for different times. The PDGF α-receptor and PDGF ß-receptor mRNA levels remained relatively stable during a 4 h treatment with Act D, suggesting half-lives of at least 4 h (Table 1). In Figure 4 this is shown for NM-9 and Mero-14. In fact both transcripts are rather stable, as even prolonged exposure (6 h) to Act D did not result in significant decreases in α-receptor levels in normal mesothelial

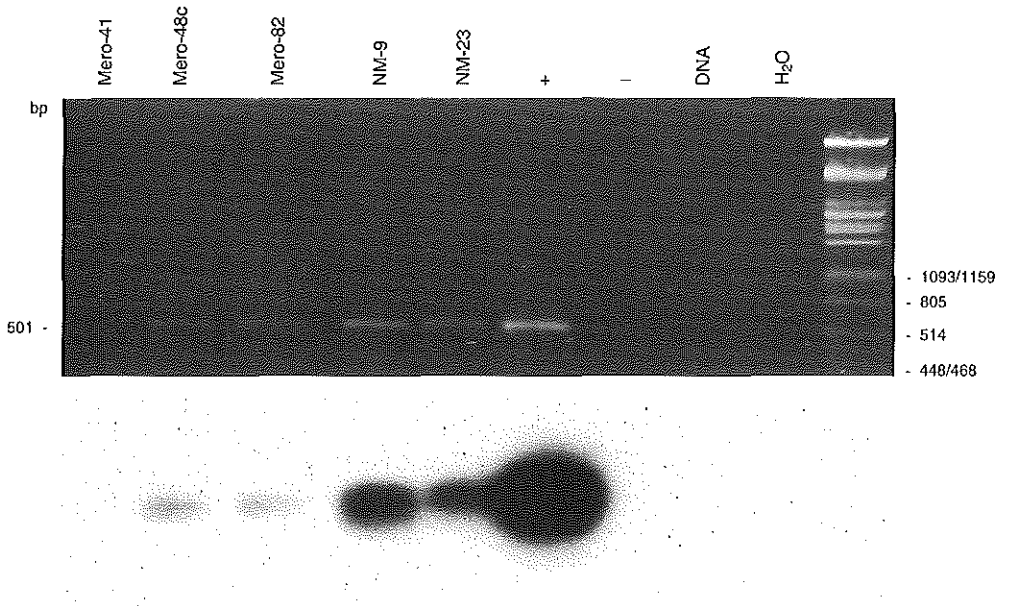

Figure 3. Reverse-transcription PCR analysis of RNA from normal mesothelial (NM-9, NM-23) and malignant mesothelioma (Mero-41, Mero-48c, Mero-82) cell lines. *Upper panel:* Ethidium bromide analysis of amplified products, using cDNA from PDGF α-receptor clone pSV7d15EB as positive control (+). PDGF ß-receptor cDNA was used to exclude cross-reactivity of the αR-7 and αR-8 primers (-). Genomic DNA and H_2O were used as negative controls. *Lower panel:* After blotting the filter was hybridized to α-receptor-specific oligo αR-3A.

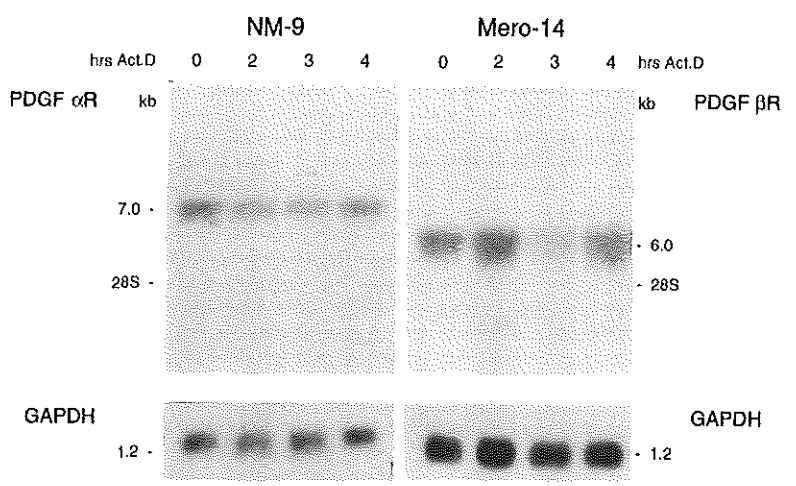

Figure 4. Northern blot analysis of total RNA from normal mesothelial (NM-9) and malignant mesothelioma (Mero-14) cell lines. Total RNA (25 µg) of NM-9 cells cultured in the presence of 5 µg/ml actinomycin D (Act D) for the indicated times was blotted and hybridized to PDGF α-receptor and GAPDH probes. Similarly, total RNA of Mero-14 cells cultured with Act D, was hybridized to PDGF ß-receptor and GAPDH probes.

cell lines or in ß-receptor levels in mesothelioma cell lines (data not shown). Further support for the high PDGF receptor mRNA stability in these cells came from the observation that at the utilized Act D concentration the PDGF A-chain messenger half-life of these cells was 2 to 3 h. This PDGF A-chain half-life is in agreement with our earlier findings (18). Unfortunately, the stability of PDGF α-receptor transcripts in malignant mesothelioma cell lines could not be determined due to the inability to detect these messengers in these cell lines by Northern blot analysis. RT-PCR of Act D-treated cells has not been performed because the quality of the RNA isolated from the Act D-treated cells was not constant enough for reliable determination of PDGF α-receptor mRNA half-lives in these cells.

Differential effects of TGF-ß1 on PDGF α- and ß-receptor mRNA levels

To further identify which factor(s) may be involved in regulation of the differential PDGF rceptor expression between normal and malignant mesothelial cells, we evaluated the possible contribution of TGF-ß1. TGF-ß1 is one of the most extensively studied regulators of PDGF receptor expression and is known to be produced by mesothelial cells. In a pilot experiment, that was designed to identify conditions for TGF-ß1 stimulation, maximal effects were seen under serum-free conditions using 2 ng/ml TGF-ß1 for 24 h (data not shown). Serum-free culture was found to slightly alter PDGF α- and ß-receptor mRNA levels in normal and malignant mesothelial cells, respectively, but no significant induction of the otherwise undetectable receptor type was seen. Based on these results, the effect of TGF-ß1 stimulation was analyzed in a panel of cell lines. Serum-free culture of normal mesothelial cell lines (NM-21 and NM-25) for an additional 24 h resulted in slightly altered

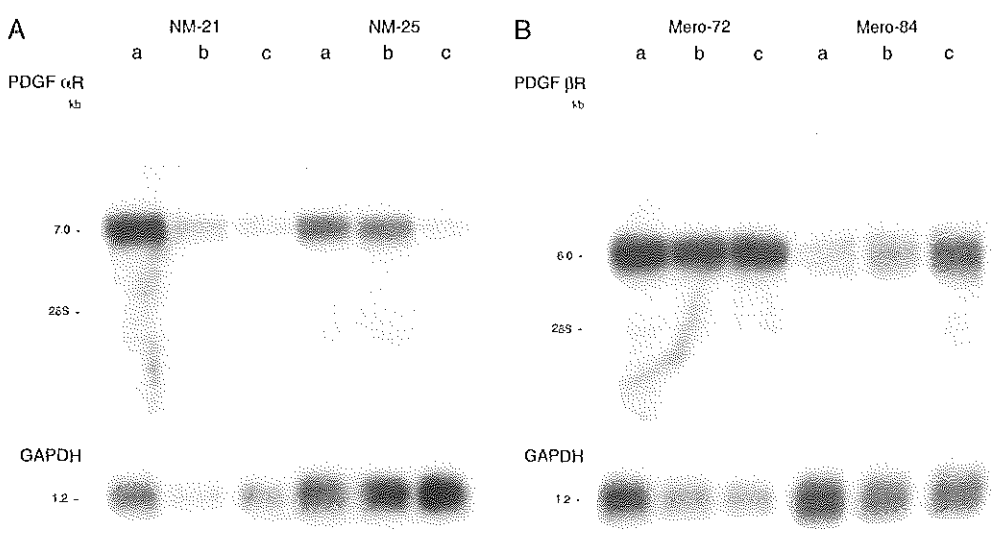

Figure 5. Northern blot analysis of total RNA from normal mesothelial (NM-21, NM-25) (A) and malignant mesothelioma (Mero-72, Mero-84) (B) cell lines. Cells were either cultured without serum (lanes a), cultured without serum for another 24 h. (lanes b) or cultured in the presence of 2 ng/ml transforming growth factor-ß1 (TGF-ß1) under serumfree conditions (lanes c). Total RNA (25 µg) was blotted and hybridized to PDGF α-receptor (A) and PDGF ß-receptor (B) probes. GAPDH hybridization was done for control.

PDGF α-receptor mRNA expression levels (Figure 5A, lanes a,b). In parallel cultures addition of 2 ng/ml TGF-ß1 caused downregulation of α-receptor messenger expression in these cell lines, as can be clearly seen when comparing lanes b and c. In malignant mesothelioma cell lines no PDGF-α receptor mRNA expression was seen under the conditions tested. The effect of serum-free culture and TGF-ß1 stimulation on PDGF ß-receptor mRNA levels in the malignant mesothelioma cell lines tested, was variable. In two out of six (Mero-82, Mero-84) an increase in PDGF ß-receptor level was seen after serum depletion, which was even further increased by TGF-ß1 stimulation (Figure 5B, lanes b, c). In Mero-72 the level was slightly increased by serum depletion, but TGF-ß1 did not have a clear additional effect (Figure 5B, lanes b, c). Of the other three cell lines tested, one did not demonstrate PDGF ß-receptor mRNA expression under all conditions (Mero-95), whereas the other two showed rather low (Mero-25, Mero-41) expression levels that were hardly affected by serum removal or TGF-ß1 stimulation (data not shown). In normal mesothelial cell lines a weak enhancing effect was seen after serum removal.

DISCUSSION

We previously reported on PDGF α-receptor mRNA expression in normal mesothelial cells, whereas on Northern blots only PDGF ß-receptor transcripts could be detected in nearly all malignant mesothelioma cell lines (30). The observed differences are not likely to be caused by the culture conditions, as addition of EGF and HC (added to normal mesothelial cells cultures) and serum removal just slightly modulated PDGF receptor mRNA levels in both normal and malignant mesothelial cell lines and, most importantly, did not cause induction of the otherwise undetectable PDGF receptor subunit ((31) and this study). Cytogenetic analysis did not demonstrate any consistent chromosomal aberration on chromosomes 4 (α-receptor) or 5 (ß-receptor) in the malignant mesothelioma cell lines (30). Furthermore, in the present study no PDGF α-receptor gene rearrangements or intragenic deletions were detected in the malignant mesothelioma cell lines by Southern blot analysis using restriction enzyme *Eco*RI (data not shown). We therefore studied transcriptional and post-transcriptional regulation of the differential PDGF receptor mRNA pattern in mesothelial cells. We conclude that the absence of clear PDGF ß-receptor mRNA levels in total RNA of normal mesothelial cell lines is probably caused by lack of transcription of this gene rather than by rapid decay of unstable messengers. In contrast, the absence of PDGF α-receptor mRNA on Northern blots of malignant mesothelioma cell lines cannot be explained similarly. Several lines of evidence suggest that malignant mesothelioma cell lines in fact are capable of transcribing the PDGF α-receptor gene. By nuclear run off analysis as well as RT-PCR PDGF α-receptor transcripts could be detected, although in the latter assay the expression level was rather low and variable as compared to normal mesothelial cells. Prolonged exposure of blots containing poly(A)$^+$ RNA of malignant mesothelioma cell lines also showed a faint PDGF α-receptor hybridization signal (data not shown). Furthermore, other investigators found PDGF α-receptor transcripts in their malignant mesothelioma cell lines using the sensitive RNase protection analysis (10). In agreement with all these findings we recently observed membrane-bound PDGF α-receptor proteins on malignant mesothelioma cells using immunostaining techniques (19). Furthermore, in

view of these data the earlier observed binding of radiolabeled ^{125}I-PDGF-AA to malignant mesothelioma cell lines should not be interpreted as background binding, but rather as specific, though low, binding of PDGF-AA to PDGF α-receptors on these cells (30). Despite the demonstration of PDGF α-receptor expression in malignant mesothelioma cell lines, it remains to be determined in future studies whether growth stimulatory pathways acting via PDGF α-receptors are really of biological importance in these cells as well.

Being one of the best studied regulators of PDGF receptor expression and being produced by both normal and malignant mesothelial cells, TGF-ß1 was evaluated for its putative contribution to the differential PDGF receptor expression in mesothelial cell lines. After stimulation with TGF-ß1, no induction of PDGF α- and ß-receptor mRNA was seen in normal and malignant mesothelial cells, respectively. However, PDGF α-receptor mRNA levels in normal mesothelial cells were found to be decreased by TGF-ß1, which is similar to what has been described for e.g. smooth muscle cells and fibroblasts (3,21). The effect of TGF-ß1 on PDGF ß-receptor expression in malignant mesothelioma cell lines was variable, which also fits in with data from other cell types, like smooth muscle and mesangial cells (12,15,21). Taken together, the effect of TGF-ß1 stimulation is not likely to explain the differential PDGF receptor expression. Nevertheless, the low PDGF α-receptor mRNA expression in malignant mesothelioma cell lines may well be (partly) the consequence of α-receptor downregulation by endogenously produced TGF-ß1. However, the latter suggestion awaits further experimental proof. Recently, it has been shown that antisense messengers against TGF-ß2 and to a lesser extent against TGF-ß1, resulted in growth-inhibition of mesotheioma cells *in vitro* and in reduced tumorigenicity and increased T-lymphocyte infiltration *in vivo* (9). Together these results suggest an important role for TGF-ß in mesothelial carcinogenesis. The results described in this study indicate that modulation of PDGF receptor mRNA expression may be another effect of TGF-ß1 in (part of the) malignant mesothelioma cells.

The apparent inability to detect PDGF α-receptor messengers in malignant mesothelioma cells by Northern blot analysis, is most probably due to rapid degradation, suggesting that the PDGF α-receptor transcript is rather unstable in malignant mesothelioma cells. In contrast, the α-receptor messenger in normal mesothelial cells is quite stable, with a half-life of at least 6 h. As inhibition of protein synthesis did not seem to influence PDGF α-receptor mRNA stability in malignant mesothelioma cells, labile degrading proteins are not likely to be involved. However, CHX-insensitive proteins may very well be responsible for the observed difference in stability. Alternatively, it may be that the absence of certain degradation-protecting proteins or the presence of mutations in 3′ untranslated sequences (UTS) cause differences in PDGF α-receptor messenger degradation between normal and malignant cells. AUUUA regions in the 3′ UTS have earlier been shown to play a role in stability of e.g. GM-CSF transcripts (2). It remains to be determined if any of these mechanisms accounts for the apparent difference in PDGF α-receptor stability between normal and malignant mesothelial cells.

Thusfar there have been, to our knowledge, no reports on promoter function and involvement of cis-acting regulatory elements in PDGF ß-receptor transcription. Concerning the PDGF α-receptor, recently a study was published in which a 93 bp minimal promoter region, relative to the transcription start, was identified, that functioned in a way that mirrored mouse tissue-specific PDGF α-receptor expression (32). Moreover, a similar though

larger promoter region was identified in human Tera-2 cells (1). It would be interesting to see whether this promoter region would be sufficient to drive PDGF α-receptor transcription in mesothelial cells as well. Based on the earlier discussed differences and similarities in regulation of PDGF α-receptor and ß-receptor expression, normal and malignant mesothelial cell lines could thus be very helpful for characterization of promoter and upstream regulatory elements of both PDGF receptor genes in human cells.

In conclusion, data on regulation of PDGF receptor expression collectively suggests that both PDGF α- and ß-receptor transcripts can be expressed by malignant mesothelioma cell lines. However, there seems to be a difference in stability between PDGF α- and ß-receptor transcripts. Moreover, α-receptor processing in malignant mesothelioma cell lines seems to be different from that in normal mesothelial cell lines as well. Further identification of the mechanism behind this processing will therefore be a topic of future studies.

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REFERENCES

1. Afink, G.B., Nistér, M., Stassen, B.H.G.J., Joosten, P.H.L.J., Rademakers, P.J.H., Bongcam-Rudloff, E., Van Zoelen, E.J.J., Mosselman, S. (1995) Oncogene 10, 1667-1672.
2. Akashi, M., Shaw, G., Gross, M., Saito, M., Koeffler, H.P. (1991) Blood 78, 2005-2012.
3. Battegay, E.J., Raines, E.W., Seifert, R.A., Bowen-Pope, D.F., Ross, R. (1990) Cell 63, 515-524.
4. Benham, F.J., Hodgkinson, S., Davies, K.E. (1984) EMBO J. 3, 2635-2640.
5. Betsholtz, C., Johnsson, A., Heldin, C.-H., Westermark, B., Lind, P., Urdea, M.S., Eddy, R., Shows, T.B., Philpott, K., Mellor, A.L., Knott, T.J., Scott, J. (1986) Nature 320, 695-699.
6. Claesson-Welsh, L., Eriksson, A., Morén, A., Severinsson, L., Ek, B., Östman, A., Betsholtz, C., Heldin, C.-H. (1988) Mol. Cell. Biol. 8, 3476-3486.
7. Claesson-Welsh, L., Eriksson, A., Westermark, B., Heldin, C.-H. (1989) Proc. Natl. Acad. Sci. USA 86, 4917-4921.
8. Dodemont, H.J., Soriano, P., Quax, W.J., Ramaekers, F., Lenstra, J.A., Groenen, M.A., Bernardi, G., Bloemendal, H. (1982) EMBO J. 1, 167-171.
9. Fitzpatrick, D.R., Bielefeldt-Ohmann, H., Himbeck, R.P., Jarnicki, A.G., Marzo, A.L., Robinson, B.W.S. (1994) Growth Fact. 11, 29-44.
10. Garlepp, M.J., Christmas, T.I., Manning, L.S., Mutsaers, S.E., Dench, J., Leong, C., Robinson, B.W.S. (1993) Eur. Resp. Rev. 3, 189-191.
11. Gerwin, B.I., Lechner, J.F., Reddel, R.R., Roberts, A.B., Robbins, K.C., Gabrielson, E.W., Harris, C.C. (1987) Cancer Res. 47, 6180-6184.
12. Haberstroh, U., Zahner, G., Disser, M., Thaiss, F., Wolf, G., Stahl, R.A.K. (1993) Am. J. Physiol. 264, F199-F205.
13. Heldin, C.-H., Bäckström, G., Östman, A., Hammacher, A., Rönnstrand, L., Rubin, K., Nistér, M., Westermark, B. (1988) EMBO J. 7, 1387-1393.
14. Heldin, C.-H. and Westermark, B. (1990) Cell Regulation 1, 555-566.
15. Janat, M.F., Liau, G. (1992) J. Cell. Physiol. 150, 232-242.
16. Kaetzel, D.M., Coyne, D.W., Fenstermaker, R.A. (1993) Biofactors 4, 71-81.
17. Kelly, J.D., Haldeman, B.A., Grant, F.J., Murray, M.J., Seifert, R.A., Bowen-Pope, D.F., Cooper, J.A., Kazlauskas, A. (1991) J. Biol. Chem. 266, 8987-8992.
18. Langerak, A.W., Dirks, R.P.H., Versnel, M.A. (1992) Eur. J. Biochem. 208, 589-596.
19. Langerak, A.W., De Laat, P.A.J.M., Van der Linden-van Beurden, C.A.J., Delahaye, M., Van der Kwast Th.H., Hoogsteden, H.C., Benner, R., Versnel, M.A. (1995) J. Pathol. in the press.
20. Maeda, J., Ueki, N., Ohkawa, T., Iwahashi, N., Nakano, T., Hada, T., Higashino, K. (1994) Clin. Exp. Immunol. 98,

319-322.
21. Paulsson, Y., Karlsson, C., Heldin, C.-H., Westermark, B. (1993) J. Cell. Physiol. 157, 97-103.
22. Raines, E.W., Dower, S.K., Ross, R. (1989) Science 243, 393-396.
23. Raines, E.W., Bowen-Pope, D.F., Ross, R. (1990) Peptide Growth Factors and their Receptors, Handbook in Experimental Pharmacology vol. 95, part 1, pp. 173-262, Springer Verlag, Heidelberg.
24. Ross, R., Bowen-Pope, D.F., Raines, E.W. (1990) Phil. Trans. R. Soc. Lond. 327, 155-169.
25. Seifert, R.A., Hart, C.E., Phillips, P.E., Forstrom, J.W., Ross, R., Murray, M.J., Bowen-Pope, D.F. (1989) J. Biol. Chem. 264, 8771-8778.
26. Shamah, S.M., Stiles, C.D., Guha, A. (1993) Mol. Cell. Biol. 13, 7203-7212.
27. Vassbotn, F.S., Östman, A., Langeland, N., Holmsen, H.A., Westermark, B., Heldin, C.-H., Nistér, M. (1994) J. Cell. Physiol. 158, 381-389.
28. Versnel, M.A., Hagemeijer, A., Bouts, M.J., Van der Kwast, Th.H., Hoogsteden, H.C. (1988) Oncogene 2, 601-605.
29. Versnel, M.A., Bouts, M.J., Hoogsteden, H.C., Van der Kwast, Th.H., Delahaye, M., Hagemeijer, A. (1989) Int. J. Cancer 44, 256-260.
30. Versnel, M.A., Claesson-Welsh, L., Hammacher, A., Bouts, M.J., Van der Kwast, Th.H., Eriksson, A., Willemsen, R., Welma, S.M., Hoogsteden, H.C., Hagemeijer, A., Heldin, C.-H. (1991) Oncogene 6, 2005-2011.
31. Versnel, M.A., Bouts, M.J., Langerak, A.W., Van der Kwast, Th.H., Hoogsteden, H.C., Hagemeijer, A., Heldin, C.-H. (1992) Exp. Cell Res. 200, 83-88.
32. Wang, C. and Stiles, C.D. (1994) Proc. Natl. Acad. Sci. USA 91, 7061-7065.
33. Westermark, B. and Heldin, C.-H. (1991) Cancer Res. 51, 5087-5092.
34. Winkles, J.A. and Gay, C.G. (1991) Bioch. Bioph. Res. Comm. 180, 519-524.
35. Xie, J., Stroumza, J., Graves, D.T. (1994) J. Immunol. 153, 378-383.

5

WT1 EXPRESSION IN MESOTHELIAL CELL LINES

CHAPTER 5.1

THE WILMS TUMOUR WT1 GENE

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WT1 tumour suppressor gene

Wilms tumour or nephroblastoma is the most common pediatric solid tumour. Approximately 1 in 10,000 children are affected by this malignancy (1,2).

Chromosome region 11p13 has been identified as one of the loci involved in Wilms tumorigenesis, through deletion analysis of WAGR (Wilms tumour, aniridia, genitourinary abnormalities, mental retardation) patients (Table 1; 3-5). Another genetic locus on chromosome 11 at band p15 seems to play a role in predisposition to Wilms tumours found in association with BWS (Beckwith-Wiedemann syndrome) (Table 1; 6,7). Furthermore, frequent LOH of chromosome 16q suggests this region to contain yet another gene involved in Wilms tumours (8). Sofar, this locus has not been further characterized.

More thorough analysis of the 11p13 region revealed the presence of deletions in a candidate gene that was subsequently cloned by three groups and was designated WT1 (9-11). The demonstration of both germline and somatic intragenic deletions in this WT1 gene in a proportion of Wilms tumour patients indicated that it is indeed a Wilms tumour predisposition gene (12-16). The presence of point mutations in the WT1 tumour suppressor gene that may disrupt or alter its function as a transcription factor (see later) were also studied. Only in a minority of sporadic unilateral and bilateral Wilms tumours such point mutations in the DNA binding zinc finger-encoding region of WT1 were reported (17-19). In Wilms tumours occurring in Denys-Drash syndrome (DDS) patients (Table 1), WT1 point mutations were observed in nearly all cases analyzed (20-24). In DDS patients these mutations are heterozygous, suggesting a dominant-negative mode of action. Such a dominant-negative effect could either result from association with wild-type WT1 proteins or from squelching of an interactive protein involved in transregulation by WT1 (25).

TABLE 1. Association of Wilms tumours with syndromes and chromosomal loci.

syndrome	locus	tumour characteristics	tumour histology
WAGR, DDS	11p13	intralobar nephrogenic rest-like	heterogeneous (entire spectrum of nephrogenesis)
BWS	11p15	perilobar nephrogenic rest-like	homogeneous (late stages of nephrogenesis)

Abbreviations used are: BWS: Beckwith-Wiedemann syndrome (characterized by prenatal and postnatal gigantism); DDS: Denys-Drash syndrome (a triad of nephropathy, pseudohermaphroditism, and Wilms tumour); WAGR: Wilms tumour, aniridia, genitourinary abnormalities, mental retardation.

Expression and possible function of the WT1 gene

Expression of the WT1 gene is seen in a limited set of tissues in developing human and mice embryos, e.g. kidney, spleen, mesothelial linings of the coelomic cavities, gonadal ridge mesothelium, Sertoli cells and granulosa cells (26-28). In adults, WT1 expression was observed in the glomerular epithelia of the kidney, Sertoli cells in the testes and ovarian granulosa cells (26-28).

All these tissues are derived from the mesoderm and have undergone a mesenchyme to epithelial transition during development. Hence, it was proposed that WT1 may be required in this transition (26). However, expression of WT1 was also seen in ectodermally-derived tissues, like parts of the spinal cord and the developing brain in embryonic mice and rats, suggesting that WT1 might have other tissue-specific roles as well (28,29).

The WT1 expression pattern strongly suggests that WT1 plays a role in regulating genes that are involved in several stages of nephrogenesis and in development of the gonads. A crucial role for WT1 in early urogenital development was established in a gene targeting model system of murine embryonic stem cells (30). In these WT1 knockout mice a failure in kidney development was observed. In addition, abnormal development of gonads, heart and mesothelium was seen. The occurrence of Wilms tumours in combination with genitourinary abnormalities in WAGR and DDS patients further supports the view that the WT1 product may have pleiotropic effects in urogenital development.

In malignancies arising from WT1-expressing tissues other than the kidney, the WT1 gene was studied as well, although the number of reports is limited. No clear indications for WT1 contribution could be found in the neoplastic counterpart of Sertoli cells and granulosa cells, commonly referred to as sex cord-stromal tumours (31) as well as in ovarian carcinomas (32), as wild-type WT1 mRNA was observed in most cases. Furthermore, there are a few reports on WT1 expression in transformed mesothelial cells (33-35). These authors observed WT1 mRNA expression in a single normal and several malignant mesothelial cell lines. WT1 protein expression was also observed in malignant mesothelioma tumour samples (36). In a single and exceptional case of peritoneal mesothelioma a point mutation was found in the WT1 transregulating domain (33). Point mutations could not be observed in the analyzed exons of the coding region of the WT1 gene in several pleural mesotheliomas (33,34). The fact that malignant mesothelioma was mentioned as a second risk tumour in a small number of patients that had recovered from Wilms tumour (37), may indicate the possible involvement of a similar underlying genetic event in at least a subset of cases. A genetically altered WT1 gene could be a good candidate.

Regulation of WT1 expression

In several studies the WT1 gene was shown to contain a TATA-less, CCAAT-less promoter with a high (> 60%) GC-content with potential Egr-1/WT1 and Sp1 binding sites (38,39). One major and several minor WT1 transcription start sites were identified (39). The WT1 minimal promoter region was found to function in all cell types tested (40). Transactivation of a 3'-enhancer sequence at roughly 50 kb of the transcription start by GATA-1 seems to confer an additional tissue-specific increase in WT1 expressi-

on (40,41). Due to its action as a repressor of transcription, the WT1 gene can be negatively autoregulated through multiple WT1 binding sites in the WT1 promoter region (42,43). The WT1 5′ flanking promoter region appears to act as a bidirectional promoter, being involved in transcription of the Wit-1 gene in the opposite direction as well (42). The function of this Wit-1 gene product is not clear yet, as there seems to be no large open reading frame. It has been suggested to play a role in regulation of WT1 transcription (44).

The WT1 gene can express four different mRNAs through alternative splicing at two distinct sites (45,46), whereas RNA editing at position 839 further extends the number of distinct messengers (47). At the one site alternative splicing leads to insertion of 17 amino acids N-terminal of the zinc-finger region, whereas at the other site three amino acids (lysine, serine, threonine; KTS), are inserted between the 3rd and 4th zinc-finger. There is little variation in the relative distribution of these 4 splice variants among WT1-expressing Wilms tumours and fetal kidney tissue (45,46). The variant containing the extra 17 and three amino acids inserts seems to be slightly predominant. The presence of these four transcripts in all tissues studied thus far, suggests that each WT1 isoform may have an important function. This is supported by the observation that a WT1 mutation in a DDS patient affected the splice site between the 3rd and 4th zinc-finger. This resulted in a disturbed ratio of WT1 (+KTS) and WT1 (-KTS) proteins, as from one allele no WT1 (+KTS) protein could be produced (22).

Transcription regulating activity of WT1

The WT1 product shows DNA binding and transcription regulating capacity, which is in agreement with its nuclear localization in immunofluorescence analysis (48,49). The C-terminal part of the WT1 (-KTS) protein contains four zinc-fingers, which bind to DNA sequences with the core consensus element 5′-GCGGGGGCG-3′ (50). This core sequence is also recognized by the transcription factor Egr-1, that shares extensive amino acid homology to WT1 (51). WT1 exerts its transcriptional activity through the N-terminal glutamine- and proline-rich region (52).

Using a whole genome PCR-based approach it was shown that the WT1 (+KTS) isoform did not bind to the Egr-1 consensus binding site, but to other (consensus) DNA sequences in perhaps distinct target genes (53). It has been suggested, although there is no formal proof, that the recently described TCC motif in the promoters of several genes (PDGF A-chain, c-*myc*, TGF-ß3 and Ki-*ras*) could be recognized by this WT1 (+KTS) isoform (54). The TCC motif functioned in a way similar to the previously mentioned 5′-GCG GGG GCG-3′ Egr-1 binding site. Using purified protein, Drummond et al. (55) recently demonstrated the DNA binding specificities of the WT1 (+KTS) variant; a 12 bp-recognition element seems to be bound by all four WT1 zinc-fingers together, thus conferring more sequence specificity and possibly extending binding capacity. Others suggested that the 17 amino acids insert could act as a repressor element itself as well (56). In a recent paper Larsson et al. (57) showed that alternative splicing within the WT1 zinc-finger region resulted in different subnuclear localizations of the WT1 protein. The WT1 (+ KTS) isoforms mainly localized with splicing factors suggesting a role in post-transcriptional regulation of mRNA levels, whereas the (−KTS) isoform was found with DNA and transcription factors. Together these findings suggest

distinct functions for the WT1 isoforms.

In transient transfection studies the IGF2, PDGF A-chain, IGFIR, CSF1, TGF-ß, RAR-α1, and Pax-2 genes have been identified as potential target genes for repression of transcription by the WT1 (-KTS) isoform (58-65). These results have led to the hypothesis that WT1 functions as a tumour suppressor gene product and that reduction or absence of WT1 expression which is seen in some cases of Wilms tumour, results in a higher expression of important growth factor and growth factor receptor genes. The observation that in Wilms tumours IGF2 was found to be overexpressed compared to normal kidneys confirmed this suggestion (66,67). However, the transcription repressing ability of WT1 was based on transfection studies in which promoters of target genes upstream of a reporter gene were found to be influenced by WT1. So far no data have been published on the regulation by WT1 of endogenous levels of any of these target genes in WT1-expressing cells.

Recently, transcriptional regulation by WT1 was found to be more complicated, as transactivation by WT1 was also observed. Maheswaran and coworkers (68) demonstrated transactivation of an Egr-1 promoter construct in the absence of functional p53, whereas repression was seen in the presence of wild-type p53. An intriguing observation is the presence of a point mutation in the transactivating domain of the remaining WT1 allele in a Wilms tumour arising in a child with WAGR syndrome. This mutation caused the conversion of the WT1 protein from a transcriptional repressor into a transcriptional activator (69). It has been suggested that this may be caused by disruption of binding between WT1 and p53 (69). Furthermore, WT1 was shown to require binding sites 5' and 3' of the transcription start site of the PDGF A-chain promoter in order to repress transcription, whereas the presence of only one of these binding sites resulted in transactivation by WT1 (70). A totally distinct mechanism of regulation by WT1 was observed in a Wilms tumour cell line expressing an abnormal WT1 transcript. This transcript, that was also observed in varying amounts in several Wilms tumour samples, was devoid of exon 2-encoded sequence, which caused WT1 to switch from a transrepressor to a transactivator in Egr-1-driven reporter studies (71).

In Chapter 5.2 the transregulatory activities of WT1 are evaluated using mesothelial cell lines as a model system.

REFERENCES

1. Van Heyningen V, Hastie ND. Wilms' tumour: reconciling genetics and biology. Trends Genet. 8, 16-21, 1992.
2. Haber DA, Housman DE. Role of the WT 1 gene in Wilms' tumour. Cancer Surv. 12: 105-117, 1992.
3. Riccardi VM, Sujanski E, Smith AC, Francke U. Chromosomal imbalance in the aniridia-Wilms' tumor association: 11p interstitial deletion. Pediatrics 61: 604-610, 1978.
4. Francke U, Holmes LB, Atkins L, Riccardi VM. Aniridia-Wilms' tumour association: evidence for specific deletion of 11p13. Cytogenet. Cell Genet. 24: 185-192, 1979.
5. Van Heyningen V, Boyd PA, Seawright A, Fletcher JM, Fantes JA, Buckton KE, Spowart G, Porteous DJ, Hill RE, Newton MS, Hastie ND. Molecular analysis of chromosome 11 deletions in aniridia-Wilms' tumour syndrome. Proc. Natl. Acad. Sci. USA 82: 8592-8596, 1985.
6. Grundy P, Koufos A, Morgan K, Li FP, Meadows AT, Cavenee WK. Familial predisposition to Wilms' tumour does not map to the short arm of chromosome 11. Nature 336: 374-376, 1988.

7. Huff V, Compton DA, Chao LY, Strong LC, Geiser CF, Saunders GF. Lack of a linkage of familial Wilms' tumour to chromosomal band 11p13. Nature 336: 377-378, 1988.
8. Maw MA, Grundy PE, Millow LJ, Eccles MR, Dunn RS, Smith PJ, Feinberg AP, Law DJ, Paterson MC, Telzerow PE, Callen CF, Thompson AD, Richards RI, Reeve AE. A third Wilms' tumor locus on chromosome 16q. Cancer Res. 52: 3094-3098, 1992.
9. Bonetta L, Kuehn SE, Huang A, Law DJ, Kalikin LM, Koi M, Reeve AE, Brownstein BH, Yeger H, Williams BRG, Feinberg AP. Wilms tumor locus on 11p13 defined by multiple CpG island-associated transcripts. Science 250: 994-997, 1990.
10. Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, Rose EA, Kral A, Yeger H, Lewis W, Jones C, Housman DE. Isolation and characterization of a zinc-finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell 60: 509-520, 1990.
11. Gessler M, Poustka A, Cavenee W, Neve RL, Orkin SH, Bruns GAP. Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. Nature 343: 774-778, 1990.
12. Haber DA, Buckler AJ, Glaser T, Call KM, Pelletier J, Sohn RL, Douglass EC, Housman DE. An internal deletion within an 11p13 zinc finger gene contributes to the development of Wilms' tumor. Cell 61: 1257-1269, 1990.
13. Cowell JK, Wadey RB, Haber DA, Call KM, Housman DE, Pritchard J. Structural rearrangements of the WT 1 gene in Wilms' tumour cells. Oncogene 6: 595-599, 1991.
14. Huff V, Miwa H, Haber DA, Call KM, Housman DE, Strong L, Saunders GF. Evidence for WT 1 as a Wilms tumor (WT) gene: intragenic germinal deletion in bilateral WT. Am. J. Hum. Genet. 48: 997-1003, 1991.
15. Brown KW, Watson JE, Poirier V, Mott MG, Berry PJ, Maitland NJ. Inactivation of the remaining allele of the WT 1 gene in a Wilms' tumour from a WAGR patient. Oncogene 7: 763-768, 1992.
16. Tadokoro K, Fujii H, Ohshima A, Kakizawa Y, Shimizu K, Sakai A, Sumiyoshi K, Inoue T, Hayashi Y, Yamada M. Intragenic homozygous deletion of the WT 1 gene in Wilms' tumor. Oncogene 7: 1215-1221, 1992.
17. Little MH, Prosser J, Condie A, Smith PJ, Van Heyningen V, Hastie ND. Zinc finger point mutations within the WT 1 gene in Wilms tumor patients. Proc. Natl. Acad. Sci. USA 89: 4791-4795, 1992.
18. Coppes MJ, Liefers GJ, Paul P, Yeger H, Williams BRG. Homozygous somatic WT 1 point mutations in sporadic unilateral Wilms' tumor. Proc. Natl. Acad. Sci. USA. 90: 1416-1419, 1993.
19. Varanasi R, Bardeesy N, Ghahremani M, Petruzzi M-J, Nowak N, Adam MA, Grundy P, Shows TB, Pelletier J. Fine structure analysis of the WT 1 gene in sporadic Wilms' tumors. Proc. Natl. Acad. Sci. USA 91: 3554-3558, 1994.
20. Pelletier J, Bruening W, Kashtan CE, Mauer SM, Manivel JC, Striegel JE, Houghton DC, Junien C, Habib R, Fouser L, Fine RN, Silverman BL, Haber DA, Housman D. Germline mutations in the Wilms' tumour suppressor gene are associated with abnormal urogenital development in Denys-Drash syndrome. Cell 67: 437-447, 1991.
21. Baird PN, Santos A, Groves N, Jadresic L, Cowell JK. Constitutional mutations in the WT 1 gene in patients with Denys-Drash syndrome. Hum. Mol. Genet. 1: 301-305, 1992.
22. Bruening W, Bardeesy N, Silverman BL, Cohn RA, Machin GA, Aronson AJ, Housman D, Pelletier J. Germline intronic and exonic mutations in the Wilms' tumour gene (WT 1) affecting urogenital development. Nat. Genet. 1: 144-148, 1992.
23. Little MH, Williamson KA, Mannens M, Kelsey A, Gosden C, Hastie ND, Van Heyningen V. Evidence that WT 1 mutations in Denys-Drash syndrome patients may act in a dominant-negative fashion. Hum. Mol. Genet. 2, 259-264, 1993.
24. Ogawa O, Eccles ME, Yun K, Mueller RF, Holdaway MDD, Reeve AE. A novel insertional mutation at the third zinc-finger coding region of the WT 1 gene in Denys-Drash syndrome. Hum. Mol. Genet. 2: 203-204, 1993.
25. Wang Z-Y, Qui QQ, Gurrieri M, Huang J, Deuel TF. WT1, the Wilms' tumor suppressor gene product, represses transcription through an interactive nuclear protein. Oncogene 10: 1243-1247, 1995.
26. Pritchard-Jones K, Fleming S, Davidson D, Bickmore W, Porteous D, Gosden C, Bard J, Buckler A, Pelletier J, Housman D, Van Heyningen V, Hastie ND. The candidate Wilms' tumour gene is involved in genitourinary development. Nature 346: 194-197, 1990.
27. Pelletier J, Schalling M, Buckler AJ, Rogers A, Haber DA, Housman DE. Expression of the Wilms' tumour gene WT 1 in the murine urogenital system. Gene Dev. 5: 1345-1356, 1991.
28. Armstrong JF, Pritchard-Jones K, Bickmore WA, Hastie ND, Bard JBL. The expression of the Wilms' tumour gene, WT 1, in the developing mammalian embryo. Mech. Develop. 40: 85-97, 1992.
29. Sharma PM, Yang X, Bowman M, Roberts V, Sukumar S. Molecular cloning of rat Wilms' tumour complementary DNA and a study of messenger RNA expression in the urogenital system and the brain. Cancer Res. 52: 6407-6412, 1992.
30. Kreidberg JA, Sariola H, Loring JM, Maeda M, Pelletier J, Housman D, Jaenisch R. WT 1 is required for early kidney development. Cell 74: 679-691, 1993.

31. Coppes MJ, Ye Y, Rackley R, Zhao X-I, Liefers GJ, Casey G, Williams BRG. Analysis of WT 1 in granulosa cell and other sex cord-stromal tumours. Cancer Res. 53: 2712-2714, 1993.
32. Viel A, Gianni F, Capozzi E, Canzonieri V, Scarabelli C, Gloghini A, Boicchi M. Molecular mechanisms possibly affecting WT 1 function in human ovarian tumors. Int. J. Cancer 57: 515-521, 1994.
33. Park S, Schalling M, Bernard A, Maheswaran S, Shipley GC, Roberts D, Fletcher J, Shipman R, Rheinwald J, Demetri G, Griffin J, Minden M, Housman DE, Haber DA. The Wilms tumour gene WT 1 is expressed in murine mesoderm-derived tissues and mutated in a human mesothelioma. Nat. Genet. 4: 415-420, 1993.
34. Quek HH, Chow VTK, Tock EPC. The third zinc finger of the WT 1 gene is mutated in Wilms' tumour but not in a broad range of other urogenital tumours. Anticancer Res. 13: 1575-1580, 1993.
35. Walker C, Rutten F, Yuan X, Pass H, Mew DM, Everitt J. Wilms' tumor suppressor gene expression in rat and human mesothelioma. Cancer Res. 54: 3101-3106, 1994.
36. Amin KM, Litzky LA, Smythe WR, Mooney AM, Morris JM, Mews DJY, Pass HI, Kari C, Rodeck U, Rauscher FJ III, Kaiser LR, Albelda SM. Wilms' tumor 1 susceptibility (WT1) gene products are selectively expressed in malignant mesothelioma. Am. J. Pathol. 146: 344-356, 1995.
37. Austin MB, Fechner RE, Roggli VL. Pleural malignant mesothelioma following Wilms' tumor. Am. J. Clin. Pathol. 86: 227-230, 1986.
38. Gessler M, Bruns GAP. Sequence of the WT 1 upstream region including the Wit-1 gene. Genomics 17: 499-501, 1993.
39. Hofmann W, Royer H-D, Drechsler M, Schneider S, Royer-Pokora B. Characterization of the transcriptional regulatory region of the human WT 1 gene. Oncogene 8: 3123-3132, 1993.
40. Fraizer GC, Wu Y-J, Hewitt SM, Maity T, Ton CCT, Huff V, Saunders GF. Transcriptional regulation of the human Wilms' tumor gene (WT 1). J. Biol. Chem. 269: 8892-8900, 1994.
41. Wu Yj, Fraizer GC, Saunders GF. GATA-1 transactivates the WT1 hematopoietic specific enhancer. J. Biol. Chem. 270: 5944-5949, 1995.
42. Campbell CE, Huang A, Gurney AL, Kessler PM, Hewitt JA, Williams BRG. Antisense transcripts and protein binding motifs within the Wilms tumour (WT 1) locus. Oncogene 9: 583-595, 1994.
43. Rupprecht HD, Drummond IA, Madden SL, Rauscher FJ III, Sukhatme VP. The Wilms' tumor suppressor gene WT 1 is negatively autoregulated. J. Biol. Chem. 269: 6198-6206, 1994.
44. Eccles MR, Grubb G, Ogawa O, Szeto J, Reeve AE. Cloning of novel Wilms tumor gene (WT1) cDNAs; evidence for antisense transcription of WT1. Oncogene 9: 2059-2063, 1994.
45. Haber DA, Sohn RL, Buckler AJ, Pelletier J, Call KM, Housman DE. Alternative splicing and genomic structure of the Wilms' tumor gene WT 1. Proc. Natl. Acad. Sci. USA 88: 9618-9622, 1991.
46. Brenner B, Wildhardt G, Schneider S, Royer-Pokora B. RNA polymerase chain reaction detects different levels of four alternatively spliced WT 1 transcripts in Wilms' tumors. Oncogene 7: 1431-1433, 1992.
47. Sharma PM, Bowman M, Madden SL, Rauscher FJ III, Sukumar S. RNA editing in the Wilms' tumor susceptibility gene, WT 1. Genes Dev. 8: 720-731, 1994.
48. Telerman A, Dodemont H, Degraef C, Galand P, Bauwens S, Van Oostveldt P, Amson RA. Identification of the cellular protein encoded by the human Wilms' tumour (WT 1) gene. Oncogene 7: 2545-2548, 1992.
49. Mundlos S, Pelletier J, Darveau A, Bachmann M, Winterpracht A, Zabel B. Nuclear localization of the protein encoded by the Wilms' tumor gene WT 1 in embryonic and adult tissues. Development 119; 1329-1341, 1993.
50. Rauscher FJ III, Morris JF, Tournay OE, Cook DM, Curran T. Binding of the Wilms' tumor locus zinc-finger protein to the EGR-1 consensus sequence. Science 250: 1259-1262, 1990.
51. Sukhatme VP, Cao X, Chang LC, Tsai-Morris CH, Stamenkovich D, Ferreira PCP, Cohen DR, Edwards SA, Shows TB, Curran T, LeBeau MM, Adamson ED. A zinc-finger encoding gene coregulated with *c-fos* during growth and differentiation and after cellular depolarization. Cell 53: 37-43, 1988.
52. Madden SL, Cook DM, Morris JF, Gashler A, Sukhatme VP, Rauscher FJ III. Transcriptional repression mediated by the WT 1 Wilms tumor gene product. Science 253: 1550-1553, 1991.
53. Bickmore WA, Oghene K, Little MH, Seawright A, Van Heyningen V, Hastie ND. Modulation of DNA binding specificity by alternative splicing of the Wilms' tumour WT 1 gene transcript. Science 257: 235-237, 1992.
54. Wang ZY, Qiu QQ, Enger KT, Deuel TF. A second transcriptionally active DNA-binding site for the Wilms' tumour gene product, WT 1. Proc. Natl. Acad. Sci. USA 90: 8896-8900, 1993.
55. Drummond IA, Rupprecht HD, Rohwer-Nutter P, Lopez-Guisa JM, Madden SL, Rauscher FJ III, Sukhatme VP. DNA recognition by splicing variants of the Wilms' tumor suppressor, WT 1. Mol. Cell. Biol. 14: 3800-3809, 1994.
56. Wang ZY, Qiu QQ, Huang J, Gurrieri M, Deuel, T.F. Products of alternatively spliced transcripts of the Wilms' tumor suppressor gene, wt1, have altered DNA binding specificty and regulate transcription in different ways. Oncogene 10: 415-422, 1995.

57. Larsson SH, Charlieu J-P, Miyagawa K, Engelkamp D, Rassoulzadegan M, Ross A, Cuzin F, Van Heyningen V, Hastie ND. Subnuclear localization of WT1 in splicing or transcription factor domains is regulated by alternative splicing. Cell 81: 391-401, 1995.
58. Drummond IA, Madden SL, Rohwer-Nutter P, Bell GI, Sukhatme VP, Rauscher FJ III. Repression of the insulin-like growth factor II gene by the Wilms' tumor suppressor WT 1. Science 257: 674-678, 1992.
59. Gashler AL, Bonthron DT, Madden SL, Rauscher FJ III, Collins T, Sukhatme VP. Human platelet-derived growth factor A chain is transcriptionally repressed by the Wilms tumor suppressor WT 1. Proc. Natl. Acad. Sci. USA 89: 10984-10988, 1992.
60. Wang ZY, Madden SL, Deuel TF, Rauscher FJ III. The Wilms' tumor gene product WT 1 represses transcription of the platelet-derived growth factor A-chain gene. J. Biol. Chem. 267: 21999-22002, 1992.
61. Harrington MA, Konicek B, Song A, Xia XL, Fredericks WJ, Rauscher FJ III. Inhibition of colony-stimulating factor-1 promoter activity by the product of the Wilms' tumour locus. J. Biol. Chem. 268: 21271-21276, 1993.
62. Dey BR, Sukhatme VP, Roberts AB, Sporn MB, Rauscher FJ III, Kim S-J. Repression of the transforming growth factor-ß1 gene by the Wilms' tumor suppressor WT 1 gene product. Mol. Endocrinol. 8: 595-602, 1994.
63. Werner H, Rauscher FJ III, Sukhatme VP, Drummond IA, Roberts CT Jr, LeRoith D. Transcriptional repression of the the insulin-like growth factor I receptor (IGF-I-R) gene by the tumor suppressor WT 1 involves binding to sequences both upstream and downstream of the IGF-I-R gene transcription start site. J. Biol. Chem. 269: 12577-12582, 1994.
64. Goodyer P, Dehbi M, Torban E, Bruening W, Pelletier J. Repression of the retinoic acid receptor-α gene by the Wilms' tumor suppressor gene product, wt1. Oncogene 10: 1125-1129, 1995.
65. Ryan G, Steele-Perkins V, Morris JF, Rauscher FJ, Dressler GR. Repression of *Pax-2* by *WT1* during normal kidney development. Development 121: 867-875, 1995.
66. Reeve AE, Eccles MR, Wilkins RJ, Bell GI, Millow LJ. Expression of insulin-like growth factor-II transcripts in Wilms' tumour. Nature 317: 258-260, 1985.
67. Yun K, Fidler A, Eccles MR, Reeve AE. Insulin-like growth factor II and WT 1 transcript localization in human fetal kidney and Wilms' tumour. Cancer Res. 53: 5166-5171, 1993.
68. Maheswaran S, Park S, Bernard A, Morris JF, Rauscher FJ III, Hill DE, Haber DA. Physical and functional interaction between WT 1 and p53 proteins. Proc. Natl. Acad. Sci. USA 90: 5100-5104, 1993.
69. Park S, Tomlinson G, Nisen P, Haber DA. Altered trans-activational properties of a mutated WT 1 gene product in a WAGR-associated Wilms' tumour. Cancer Res. 53: 4757-4760, 1993.
70. Wang ZY, Qiu QQ, Deuel, T.F. The Wilms' tumor gene product WT 1 activates or suppresses transcription through separate functional domains. J. Biol. Chem. 268: 9172-9175, 1993.
71. Haber DA, Park S, Maheswaran S, Englert C, Re GG, Hazen-Martin DJ, Sens DA, Garvin AJ. WT 1-mediated growth suppression of Wilms' tumour cells expressing a WT 1 splicing variant. Science 262: 2057-2059, 1993.

CHAPTER 5.2

Expression of the Wilms' Tumor Gene *WT1* in Human Malignant Mesothelioma Cell Lines and Relationship to Platelet-Derived Growth Factor A and Insulin-Like Growth Factor 2 Expression

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Mutations in the *WT1* tumor suppressor gene are known to contribute to the development of Wilms' tumor (WT) and associated gonadal abnormalities. *WT1* is expressed principally in the fetal kidney, developing gonads, and spleen and also in the mesothelium, which lines the coelomic cavities. These tissues develop from mesenchymal components that have subsequently become epithelialized, and it has therefore been proposed that *WT1* may play a role in this transition of cell types. To test the possible involvement of this gene in malignant mesothelioma, we have first studied its expression in a panel of human normal and malignant mesothelial cell lines. *WT1* mRNA expression levels varied greatly between the cell lines and no specific chromosomal aberration on 11p, which could be related to the variation in *WT1* expression in these cell lines, was observed. Furthermore, no gross deletions, rearrangements, or functionally inactivating point mutations in the *WT1* coding region were identified. All four *WT1* splice variants were observed at similar levels in these cell lines. The *WT1* gene encodes a zinc-finger transcription factor and the four protein isoforms are each believed to act as transcriptional repressors of certain growth factor genes. Lack of *WT1* expression is thus predicted to result in growth stimulation of tumor cells. Binding of one particular WT1 isoform construct to the insulin-like growth factor 2 (*IGF2*) and platelet-derived growth factor A (*PDGFA*) gene promoters has been demonstrated to result in repression of these genes in transient transfection studies. Analysis of *IGF2* and *PDGFA* mRNA expression levels compared with *WT1* mRNA expression levels failed to demonstrate an inverse correlation in the mesothelial cell lines, which endogenously express these genes. Finally, the putative role of WT1 in the transition of cell types was investigated. No obvious correlation between *WT1* expression levels and cell morphology of the malignant mesothelial cell lines was evident from this study. Moreover, no change in *WT1* expression was observed in normal mesothelial cells which were, by alteration of culture conditions, manipulated to switch from the mesenchymal to epithelial morphology. *Genes Chromosom Cancer 12:87–96 (1995).* © 1995 Wiley-Liss, Inc.

INTRODUCTION

Human malignant mesothelioma is a mesodermally derived tumor, which is most often found in the pleura. Its incidence is strongly associated with exposure to asbestos fibers (Wagner et al., 1960). Malignant mesothelioma is thought to develop from mesothelial cells, which form a specialized epithelium lining the coelomic cavities. Malignant mesothelioma was mentioned as one of the so-called second risk tumors in a few patients who had recovered from the pediatric kidney malignancy Wilms' tumor (WT) (Austin et al., 1986). This suggests the possible involvement of a common underlying genetic event in both malignancies.

The *WT1* locus on 11p13 has been identified as one of the chromosomal loci contributing to WT development (Call et al., 1990; Gessler et al., 1990). *WT1* encodes a protein with DNA-binding capacity, elicited by four zinc-fingers at the C-terminal part, which bind to DNA sequences with the core consensus element 5'-GCGGGGGCG-3' (Rauscher et al., 1990). The WT1 protein additionally possesses transcriptional regulatory activity exerted through the N-terminal glutamine- and proline-rich regions of the protein (Madden et al., 1991). Alternative splicing of the *WT1* gene at two independent splice sites has been shown to result in the formation of four *WT1* splice variants. In tumor tissue and fetal kidney there appears to be little variation in the ratios of these four isoforms (Haber et al., 1991; Brenner et al., 1992).

In transient transfection studies insulin-like growth factor 2 (*IGF2*), platelet-derived growth factor A (*PDGFA*), and *IGF1R* have recently been identified as potential target genes for transcriptional repression by WT1 (Drummond et al., 1992; Gashler et al., 1992; Wang et al., 1992; Werner et

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al., 1993). These and other results have led to the hypothesis that the WT1 protein functions as a tumor suppressor gene product and that reduction or absence of *WT1* expression that is seen in a proportion of WTs results in an increased expression of certain growth factor genes. However, transactivation by WT1 was also seen, depending on the presence of wild-type TP53 protein, the number of WT1 binding sites in the promoter of the target gene, or the presence of specific missense mutations within the transregulatory domain (Maheswaran et al., 1993; Park et al., 1993a; Wang et al., 1993).

Despite these results on repression or activation of certain target genes by WT1 in transient assays, not much is known about the physiological role of WT1. It has been suggested that WT1 may play a role in mediating the shift from a mesenchymal to an epithelial phenotype, as it is expressed in the nephrogenic epithelia, in epithelial cells of the gonads, and in the mesothelium (Pritchard-Jones et al., 1990; Pelletier et al., 1991; Armstrong et al., 1992). These are all cells of mesodermal origin that have undergone the transition to the epithelial morphology. However, other cell types undergoing this transition do not express the *WT1* gene, whereas in embryonic mice distinct regions in the spinal cord and brain that are of ectodermal origin display *WT1* expression, arguing that there should be other tissue-specific roles for WT1 in development (Armstrong et al., 1992). Recently, a crucial role for *WT1* in early urogenital development was established in a model system by gene targeting in murine embryonic stem cells (Kreidberg et al., 1993). In these *WT1* knockout mice a failure in kidney development was observed. This was suggested to be caused by inhibition of inductive events leading to the formation of the metanephric kidney. In addition, abnormal development of the gonads, the heart, and the mesothelium was seen as well. Abnormalities in the phenotypes of these tissues support the idea of a role for WT1 in the mesenchymal to epithelial cell transition.

As *WT1* expression was observed in the human and mouse developing and mature mesothelium (Pritchard-Jones et al., 1990; Armstrong et al., 1992; Park et al., 1993b), we were interested to determine whether *WT1* was also expressed in its malignant counterpart as this might indicate a possible involvement of *WT1* in the pathogenesis of malignant mesothelioma. In this respect, it is worth noting that cytogenetic analysis of 40 confirmed mesothelioma patients revealed karyotypic abnormalities in several chromosomes, but only in a few cases were rearrangements in 11p seen (Hagemeijer et al., 1990). We have studied expression of the *WT1* gene in a panel of human normal and malignant mesothelial cell lines. Expression levels were related to the morphology (epithelial or mesenchymal) of the mesothelioma cells in vitro in order to clarify putative WT1 involvement in cell type transition. We also investigated whether gross alterations or point mutations could be detected at the *WT1* locus, whether the four different alternatively spliced mRNAs were present in the various normal and malignant mesothelial cell lines, and whether the *WT1* mRNA expression level in these cell lines could be correlated to their *PDGFA* and *IGF2* mRNA levels.

MATERIALS AND METHODS

Cell Lines, Growth Conditions, Characterization, and Cytogenetics

Experiments were performed using the human pleural malignant mesothelioma cell lines Mero-14, -25, -41, -48b, -48c, -72, -82, -83, -84, -95, -96, and -123 (Versnel et al., 1988, 1989) and the normal mesothelial cell lines NM-1, -4, -5, -9, and -12 (Versnel et al., 1991, 1993; Langerak et al., in preparation). All mesothelioma cell lines were derived from mesothelioma patients whose diagnosis was based on routine cytology, which was histologically or ultrastructurally confirmed. Cell lines were routinely cultured as described earlier (Versnel et al., 1988, 1989). Cytogenetic analysis was performed as described earlier (Versnel et al., 1988).

Northern and Southern Blot Analyses and Probes

Northern blotting and Southern blotting were performed as described in Langerak et al. (1992). For hybridization of Northern and Southern blots a 1.8 kb *Eco*RI *WT1* fragment derived from the WT33 cDNA was used (Call et al., 1990). Northern blots were rehybridized with the 1.3 kb *Eco*RI *PDGFA* fragment (Betsholtz et al., 1986), the 1.4 kb *Xba*I-*Eco*RI *IGF2* fragment from pIGF-II (Jansen et al., 1985), and the 0.7 kb *Eco*RI-*Pst*I glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) fragment (Benham et al., 1984). Rehybridization of Southern blots was performed with a 1.3 kb *Pst*I *IGF2* fragment from pKT218 (Jansen et al., 1985).

Reverse Transcription (RT) Polymerase Chain Reaction (PCR) Analysis

RT of RNA, isolated from the normal and malignant mesothelial cell lines, and subsequent PCR

analysis were performed as described previously (Langerak et al., 1992). For amplification of the alternative splice variants the sense primers B297 (5'-TTG GTC GAC ATG ACC TGG AAT CAG/C ATG-3'; located in *WT1* exon 4) or B439 (5'-CTT GTA CGG TCG GCA TCT-3'; located in *WT1* exon 7) were used in combination with antisense primer B298 (5'-TGC AAG CTT CAG CTG AAG GGT/C TTC/T TC-3'; located in *WT1* exon 10) (Little et al., 1992). Thirty-five cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C were performed. PCR products were analyzed on 10% polyacrylamide gels using *Pst*I digested lambda DNA as a marker.

Point Mutation Analysis

Chemical cleavage mismatch analysis using hydroxylamine and osmium tetroxide (HOT analysis) was performed as described (Cotton et al., 1988; Prosser et al., 1990). Templates were generated by RT-nested PCR (Hanson et al., 1993), a strategy required due to low yields of available RNA. The *WT1* region encoding the transregulatory domain was amplified with outer primers D609 sense (5'-CAA ACA GGA GCC GAG CTG G-3') and D610 antisense (5'-GCA CAT CCT GAA TGC CTC TG-3') followed by inner primers 1 and 3 (Brown et al., 1992). The DNA-binding domain was similarly amplified with primers C582 sense (5'-AAA TGG ACA GAA GGG CAG AGC-3') and C583 antisense (5'-TTG GAA GTT GGA TGA AGA AGA TC-3') followed by primers 2 and 4 (Brown et al., 1992). PCR conditions for D609/D610 are step 1, 1 min at 94°C; step 2, 30 cycles of 30 sec at 94°C, 1 min at 54°C, and 1–2 min at 72°C; step 3, 10 min at 72°C. Touchdown PCR conditions for C582/C583 are step 1, 1 min at 94°C; step 2, 30 cycles of 30 sec at 92°C, 1 min at 62–57°C and 2 min at 72°C; step 3, 10 min at 72°C. Samples generating cleaved fragments after HOT analysis were subjected to a second round of RT-nested PCR. Then the products were purified by β-Agarase I (New England Biolabs, Beverly, MA) and subsequently sequenced directly as described (Winship, 1989).

RESULTS

WT1 mRNA Expression in Mesothelial Cell Lines

Expression of the *WT1* gene was studied on Northern blots, containing total RNA from normal and malignant mesothelial cell lines of human origin. *WT1* mRNA was found to be consistently expressed in the normal mesothelial cell lines, although some variation in the levels was observed (Fig. 1). The *WT1* expression level was highly variable in the investigated panel of malignant mesothelioma cell lines, ranging from very high (Mero-25) to nearly or totally undetectable (Mero-41, -72, -82, -83, -95) (Fig. 1). *WT1* mRNA expression levels were normalized over *GAPDH* mRNA levels after quantification by densitometric analysis (see Table 1).

Based on their histology, malignant mesotheliomas can be classified as epithelial, mesenchymal, or biphasic. The morphology of the cultured mesothelioma cells in monolayer can differ from the primary tumor-tissue morphology (see Table 1). In an attempt to clarify the possible role of the *WT1* gene product in the transition of certain cell types from a mesenchymal toward an epithelial morphology, *WT1* mRNA expression levels in the cultured mesothelioma cells were therefore related to the epithelial or fibrous/mesenchymal morphology of these cell lines. Although the highest *WT1* mRNA level was observed in Mero-25, which has the clearest epithelial phenotype, *WT1* expression was not completely confined to cell lines showing an epithelial or biphasic morphology. *WT1* mRNA was undetectable in several cell lines with a fibrous morphology like Mero-41 and -72, but other fibrous cell lines did express *WT1* (Mero-83, -96, and -123). In cell lines that were predominantly either fibrous or epithelial, expression was observed occasionally (Mero-48b, -48c, and -84), and in others not at all (Mero-82 and -95).

The putative involvement of *WT1* in the mesenchymal to epithelial shift was further studied using a second approach. It has been described that cultured normal mesothelial cells can adopt a mesenchymal (fibrous) or epithelial cell shape depending on the presence or absence of epidermal growth factor (EGF) in the culture medium, respectively (Connell and Rheinwald, 1983). Therefore, the *WT1* mRNA level was determined in two normal mesothelial cell lines under these conditions. The two cell lines showed a more epithelial morphology upon removal of EGF [and hydrocortisone (HC)] for 3 days from the standard culture medium. However, analysis of RNA isolated from the mesothelial cells cultured for 72 hr in the absence of EGF and HC did not result in a significantly altered level of *WT1* mRNA (data not shown).

Alternative Splicing Pattern of *WT1*

The *WT1* gene is capable of producing four different mRNAs by alternative splicing, which can lead to the insertion of an extra 51 bp (exon 5) upstream of the zinc-fingers and/or 9 bp (giving rise

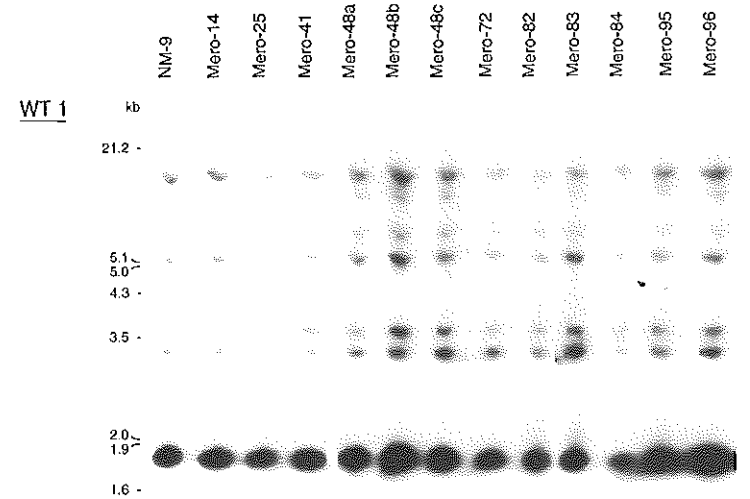

Figure 1. Northern blot analysis with 25 μg total RNA from normal (NM) and malignant (Mero) mesothelial cell lines. RNA was hybridized to *WT1* and *GAPDH* probes.

TABLE 1. Summary of Cytogenetics, Tumor Cell Line Morphology, and *WT1* Expression in Normal (NM) and Malignant (Mero) Mesothelial Cell Lines

Cell line	Tumor cell line morphology	Modal chromosome No.	No. of copies of chromosome (arm)			*WT1* mRNA level[a]	*WT1* gene structure (Southern blot)	*WT1* gene (mutations)	*WT1* mRNA (alternative splicing)
			Normal No. 11	Rearranged No. 11p	Total No. 11p				
NM-1	—	46	2	—	2	0.81	ND[b]	ND	4 isoforms
NM-4	—	46	2	—	2	0.28	ND	ND	ND
NM-5	—	46	2	—	2	0.12	ND	ND	ND
NM-9	—	46	2	—	2	0.26	Normal	ND	ND
NM-12	—	46	2	—	2	0.08	ND	ND	ND
Mero-14	Fibrous	75	3 (4)	—	3 (4)	0.04	Normal	Not found	4 isoforms
Mero-25	Epithelial	67	3 (4)	1 × inv (11)(p11q14)	4 (5)	9.59	Normal	Not found	4 isoforms
Mero-41	Fibrous	72	4	—	4	0	Normal	ND	NA[c]
Mero-48a	Epithelial (fibrous)[d]	71–75	3	1 × mar (t(9p;11p))	4	ND	Normal	Not found	ND
Mero-48b	Fibrous (epithelial)	71–75	3	1 × mar (t(9p;11p))	4	0.43	Normal	Neutral transition	ND
Mero-48c	Fibrous (epithelial)	71–75	3	1 × mar (t(9p;11p))	4	0.12	Normal	Neutral transition	4 isoforms
Mero-72	Fibrous	42	2	—	2	0	Normal	ND	NA
Mero-82	Fibrous (epithelial)	49	2	—	2	0	Normal	ND	NA
Mero-83	Fibrous	75–85	2	2 × add 11p15	4	0.04	Normal	Not found	4 isoforms
Mero-84	Fibrous (epithelial)	38	0	t(6p + ;11p−), + der(11)t(11;22)	2	0.14	Normal	Not found	4 isoforms
Mero-95	Biphasic	54–58	3	—	3	0	Normal	ND	NA
Mero-96	Fibrous	72–78	2 (3)	2 × 6q−(6p::11p)	4 (5)	0.26	Normal	Not found	4 isoforms
Mero-123	Fibrous	55	3	—	3	0.91	ND	Neutral transition	4 isoforms

[a]Shown over *GAPDH* expression levels as determined by densitometry.
[b]ND, not determined.
[c]NA, not applicable.
[d]In parentheses: minority of the cells.

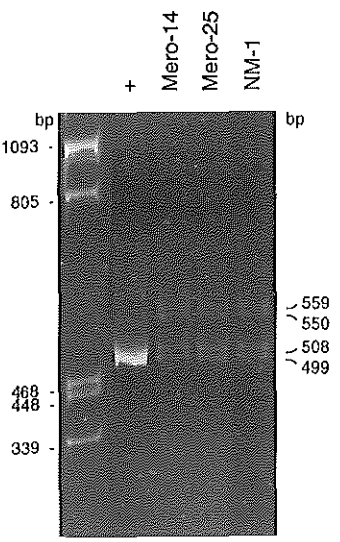

Figure 2. RT PCR analysis with RNA from malignant mesothelioma cell lines Mero-14 and -25 and normal mesothelial cell line NM-1. WT33 cDNA was used as a positive control (+). In the left lane *Pst*I digested lambda DNA was loaded as a size marker.

to the KTS amino acid sequence) between zinc-fingers 3 and 4 (Haber et al., 1991; Brenner et al., 1992). In this study the occurrence of these distinct *WT1* mRNA forms was examined in clonal cell lines. For this purpose cDNA derived from total RNA of several of the *WT1* expressing malignant mesothelioma cell lines (see Table 1) was used in a PCR reaction with primers B297 and B298. Four distinct DNA fragments of 499 bp (−51 bp, −KTS), 508 bp (−51 bp, +KTS), 550 bp (+51 bp, −KTS), and 559 bp (+51 bp, +KTS), resulting from two independent alternative splicing events, were observed (see Table 1). All four splice variants were detected in normal mesothelial cell line NM-1 as well. In Figure 2 these results are shown for the cell lines NM-1 and Mero-14 and -25. Using primers B439 and B298 in all cell lines studied, two distinct fragments of 320 and 329 bp were seen, which result from the 9 bp alternative splicing event (data not shown). In our *WT1* mRNA expressing mesothelial cell lines, little variation was observed in the ratios of the four isoforms.

Cytogenetic and Genomic Data Concerning *WT1*

The variation in *WT1* mRNA expression that we observed in the malignant mesothelioma cell lines could be due to differences in the whole or partial copy number of chromosome arm 11p. Furthermore, rearrangements of the *WT1* gene or gene amplification may also be involved. To see if any of these possibilities may explain the variation in *WT1* mRNA level in the mesothelioma cell lines, cytogenetic analysis and Southern blotting were employed.

Cytogenetic data from the mesothelioma cell lines were obtained by studying metaphase cells. Analysis of chromosome arm 11p in the mesothelioma cell lines did not point toward any specific chromosomal aberration that could be correlated to their *WT1* mRNA expression level (see Table 1). The aneuploidy of chromosome arm 11p and the *WT1* mRNA level in the various cell lines did not correlate either (Table 1).

To see if gene rearrangements had occurred in those cell lines showing very low or undetectable amounts of *WT1* mRNA, the *WT1* gene was studied by Southern blot analysis. No differences were found in the *Eco*RI and *Hin*dIII digestion pattern of DNA from any of the malignant mesothelioma cell lines studied compared to the pattern of normal mesothelial cell line NM-9 (Fig. 3; also data not shown). This indicated that gross rearrangements in the *WT1* gene had not occurred in the malignant mesothelial cell lines and thus were not likely to be the cause of undetectable *WT1* mRNA expression in Mero-41, -72, -82, and -95.

By Southern blot analysis we did detect small differences in the intensity of the bands for the various cell lines, which could in principle be caused by variation in the number of *WT1* gene copies. However, rehybridization of the filter containing *Hin*dIII digested DNA with a probe for the *IGF2* gene, which is also located on the short arm of chromosome 11 at 11p15, showed similar differences in intensity (data not shown). This meant that the *WT1* gene was not differentially amplified in any of the cell lines. Variation in the number of copies of 11p may exist but in general the number of chromosome arm 11p largely balanced the total chromosome number, which suggests that the small differences in intensity are most probably due to small variations in loading of the gels.

WT1 Point Mutation Analysis

The HOT technique of chemical cleavage mismatch analysis was used to scan virtually the entire coding sequence of *WT1* for point mutations. Only the first 256 bp in exon 1 were omitted from this analysis, as the high GC content of this region renders it refractory to PCR amplification. The malig-

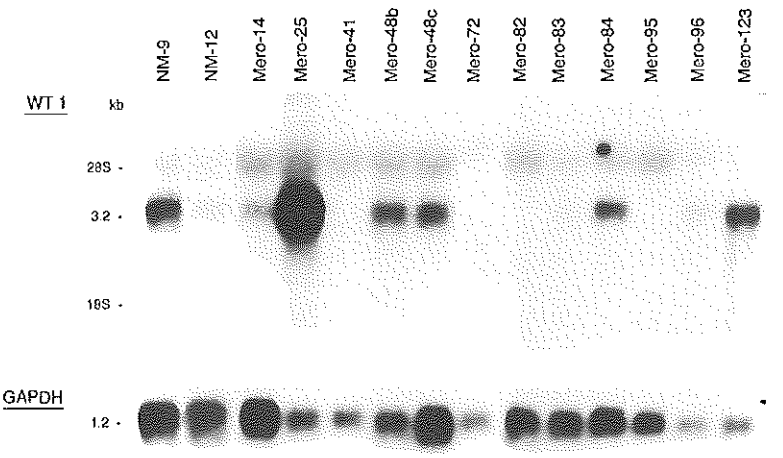

Figure 3. Southern blot analysis of the *WT1* gene. *Eco*RI-digested genomic DNA from a normal (NM-9) and several malignant (Mero) mesothelial cell lines was hybridized to a ^{32}P-labeled *WT1* probe.

nant mesothelioma cell lines Mero-14, -48b, -48c, -83, -84, and -123 were scanned for point mutations in exons 1–6, encoding the transregulatory domain, and Mero-14, -25, -48b, -48c, -83, -84, -96, and -123 were scanned for point mutations in exons 6–10, encoding the DNA-binding domain. The only base change detected was a novel C to T transition at nucleotide 768 (numbered according to Gessler et al., 1990). This exon 1 transition is a third base change and does not cause amino acid substitution (AAC/T encodes an Asn residue). It is present in the homo/hemizygous state in Mero-48b, -48c, and -123 (data not shown). No functionally inactivating nonsense or missense mutations were detected (see Table 1).

WT1 mRNA Expression in Relation to *PDGFA* and *IGF2* mRNA Expression

In transient transfection assays WT1 was reported to repress the expression of *PDGFA* and *IGF2* promoter constructs. In order to see if the described variation in *WT1* mRNA expression in our mesothelial cell lines could be related to different levels of *PDGFA* and *IGF2* mRNA, blots were rehybridized with probes for these two genes and analyzed by densitometry. The results from a representative experiment are presented as arbitrary units over *GAPDH* signals in Table 2.

TABLE 2. mRNA Expression Levels of *WT1*, *PDGFA*, and *IGF2* (6.0 and 4.8 kb) in Normal (NM) and Malignant (Mero) Mesothelial Cell Lines, Expressed as Relative Densitometric Units Over *GAPDH* Expression Levels

			IGF2	
Cell line[a]	*WT1*	*PDGFA*[b]	6.0 kb	4.8 kb
NM-1	0.81	0.65	0	0.53
NM-4	0.28	0.78	0.19	0.66
NM-9	0.26	ND[c]	0	0.23
NM-5	0.12	0.62	0.12	0.48
NM-12	0.08	ND	0.23	0.48
Mero-25	9.59	1.42	0	0.73
Mero-123	0.91	2.22	0	0.68
Mero-48b	0.43	1.41	0.28	1.02
Mero-96	0.26	3.74	1.72	6.55
Mero-84	0.14	1.55	0.02	0.23
Mero-48c	0.12	0.50	0.29	0.43
Mero-14	0.04	0.04	0	0.16
Mero-83	0.04	1.18	0.01	0.18
Mero-82	0	1.02	0	0.21
Mero-72	0	2.59	2.37	2.62
Mero-95	0	2.62	0.07	0.44
Mero-41	0	7.54	0.24	1.27

[a]Cell lines are arranged in descending order of *WT1* expression levels.
[b]Totaled levels of 2.8, 2.3, and 1.9 kb *PDGFA* transcripts.
[c]ND, not determined.

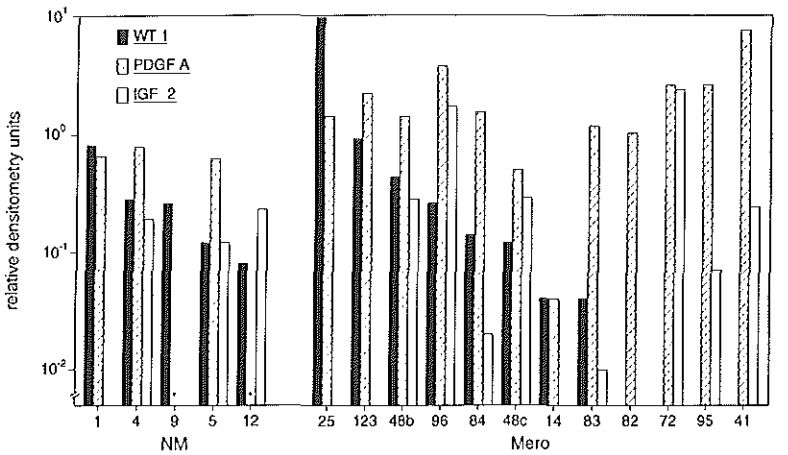

Figure 4. Relative densitometric units of the mRNA expression levels of *WT1*, *PDGFA* (2.8, 2.3, and 1.9 kb totaled) and *IGF2* (6.0 kb) in normal (NM) and malignant (Mero) mesothelial cell lines. The data are presented on a log scale. See Table 2 for the exact densitometric units. Cell lines are arranged in descending order of *WT1* expression levels. Expression levels that were not determined are indicated by an asterisk.

The densitometric data of the three *PDGFA* transcripts (2.8, 2.3, and 1.9 kb) were totaled, as expression of these three messengers is under control of a single promoter region that contains consensus WT1 binding sequences. Our mesothelioma cell lines demonstrated variation in *PDGFA* mRNA expression, but in general the expression level was higher than in normal mesothelial cell lines. In a few cell lines lacking *WT1* mRNA, a relatively high *PDGFA* expression was seen (Mero-41, -72, and -95), but other cell lines which did show *WT1* mRNA expression, i.e., Mero-96 and -123, displayed a similar *PDGFA* mRNA level. Cell line Mero-25, in which a very high *WT1* mRNA level was seen, showed an intermediate level of *PDGFA* expression. This intermediate *PDGFA* level, however, was also seen in cell lines with a lower *WT1* mRNA content than Mero-25. So, as is also illustrated in Figure 4, no clear correlation was observed between *WT1* and *PDGFA* expression in our mesothelial cell lines.

The 6.0 kb *IGF2* transcript is the product of the fetal P3 promoter, which contains WT1 consensus binding sequences, whereas the 4.8 kb transcript is expressed from a different fetal promoter (P4), also containing consensus WT1 binding sequences. Relatively high *WT1* mRNA levels were found in cell lines which do not express the 6.0 kb *IGF2* transcript (NM-1 and Mero-25 and -123), but other cell lines which demonstrated very low or no 6.0 kb *IGF2* mRNA expression, like NM-9 and Mero-14, -82, -83, -84, and -95, showed a low or intermediate *WT1* mRNA level. In the panel of normal and mesothelial cell lines no clear relationship, reciprocal or otherwise, was observed between the 6.0 kb *IGF2* and *WT1* mRNA level (see also Fig. 4). The same holds true for the expression level of the 4.8 kb *IGF2* transcript.

DISCUSSION

The WT gene *WT1* on 11p13 acquired its name, as it was originally mapped by deletion analysis of individuals with the WAGR (WT, aniridia, genitourinary abnormalities, and mental retardation) syndrome (Riccardi et al., 1978; van Heyningen et al., 1985). The demonstration of constitutional and somatic intragenic deletions in the *WT1* gene in a proportion of WT patients has confirmed that it is a WT predisposition gene (Haber et al., 1990; Cowell et al., 1991; Huff et al., 1991; Brown et al., 1992; Tadokoro et al., 1992). In expression studies in human and mouse embryos the *WT1* gene was reported to be involved in normal genitourinary development (Pritchard-Jones et al., 1990; Armstrong et al., 1992). Because of this limited spatial expression, *WT1* was suggested to be important in tissues which are of mesodermal origin and which undergo a mesenchymal to epithelial transition, although expression was also observed in the spinal cord and developing brain. In situ hybridization studies showed high *WT1* expression in the developing Sertoli cells of the testis and granulosa cells of the ovary (Pelletier et al., 1991; Armstrong et al., 1992).

As the studies by Pritchard-Jones et al. (1990) and Armstrong et al. (1992) had revealed the expression of *WT1* in the mesothelium, a specialized epithelium lining the coelomic cavities, an obvious question was if expression of the *WT1* gene could be detected in mesothelioma as well. This prompted us to study *WT1* expression in a panel of normal and malignant human mesothelial cell lines. Northern blot analysis revealed the consistent presence of *WT1* mRNA in cultured normal mesothelial cells, whereas in cultured malignant mesothelioma cells a variation in the expression level, ranging from very high to undetectable, was seen. We also found that the apparent lack of *WT1* mRNA expression in several of these cell lines probably was not due to deletions or rearrangements in the *WT1* gene. Furthermore, differences in the *WT1* mRNA expression level between different cell lines could not be accounted for by gene amplification or a specific chromosomal aberration on 11p. Differences in transcription initiation or RNA degradation thus most probably account for the variation in *WT1* mRNA expression between the malignant mesothelioma cell lines.

When the *WT1*-expressing malignant mesothelioma cell lines were analyzed for more subtle alterations within the coding sequence, no nonsense or missense mutations were found. Three lines contained an identical C to T transition in the sequence encoding the transregulatory domain. However, this mutation is predicted to be silent at the protein level and therefore most likely pathologically insignificant. Recently a homozygous *WT1* missense mutation that alters a Ser residue in the transregulatory domain has been reported for a single case of human peritoneal mesothelioma (Park et al., 1993b). This case is unusual in that the mesothelioma was not asbestos-related and was not actually a malignant tumor but rather a developmental abnormality. In addition, Park et al. (1993b) found no *WT1* mutations in 32 specimens of asbestos-related mesothelioma. For our samples it is possible that there are undetected *WT1* mutations in the 5′-most coding region of exon 1 or in the untranslated or intronic sequences of the gene. The cell lines that fail to show *WT1* mRNA expression may additionally have mutations in the promoter/control regions of the gene. Differences in the occurrence of the four alternative splicing products, which may result in altered specificity for DNA binding sites (Bickmore et al., 1992), were not observed in our panel of *WT1* expressing mesothelial cell lines. All four variants were identified earlier in WT tissue and in fetal kidney (Haber et al., 1991; Brenner et al., 1992). In these tissues the transcripts with the 9 bp alternative splice were suggested to be slightly predominant, whereas in our mesothelial cell lines we did not observe this, but a more quantitative assay has to be performed to unravel this putative discrepancy.

Taken together, the results obtained in our panel of malignant mesothelioma cell lines thus suggest that the *WT1* gene may play a role as a tumor suppressor gene in a minority of human mesotheliomas. To test this in vivo, we started to study primary tumor material from mesothelioma patients. *WT1* mRNA expression could be observed in cells from pleural effusions of four malignant mesothelioma patients (data not shown). These pleural fluids, however, contain tumor cells in combination with several other cell types, which means that this expression cannot be simply attributed to tumor cells, even though pleural fluids with a high percentage of mesothelioma cells were analyzed. Immunofluorescence staining with WT1 antibodies and/or RNA in situ hybridization would be more informative in this respect.

As the ovarian surface epithelium is considered to be a specialized mesothelium (Papadaki and Beilby, 1971) and several ovarian carcinoma cell lines demonstrated a comparable *PDGFA* and *PDGFB* mRNA expression to malignant mesothelioma cell lines (Versnel et al., 1994), we also analyzed several ovarian carcinoma cell lines for *WT1* mRNA expression. Comparable to the expression in malignant mesothelioma cell lines, in three of six serous ovarian carcinoma cell lines studied, *WT1* expression was observed on Northern blots, while in the other three no *WT1* transcripts were detected (data not shown). Furthermore, we recently observed a very high *WT1* mRNA level in cell line COV-434, which is derived from a granulosa tumor of the ovary and thus is not of mesothelial origin (data not shown). This expression is in agreement with the observed *WT1* expression in ovarian granulosa cells (Armstrong et al., 1992).

Malignant mesotheliomas are classified as epithelial, fibrous/mesenchymal, or biphasic. We therefore tried to correlate the morphology of the various malignant mesothelioma cell lines with their *WT1* mRNA expression level. Although the highest expression was found in cell line Mero-25, which has the most obvious epithelial morphology, no clear correlation could be observed between *WT1* mRNA expression and morphology. Moreover, normal mesothelial cells which can switch in morphology depending on the addition or removal of EGF from the culture medium, showed similar

WT1 expression levels independent of the phenotype of the cells. So in these mesothelial cell lines no evidence could be found for a WT1 role in the mesenchymal to epithelial transition of cells. However, this may be different in vivo. Therefore, mesothelioma tissue from patients with a biphasic (i.e., with mesenchymal and epithelial elements) malignant mesothelioma should be studied with WT1 antibodies or by RNA in situ hybridization to investigate the putative correlation between *WT1* expression and morphology in vivo.

It has been shown in transient transfection assays that WT1 represses *PDGFA* (Gashler et al., 1992; Wang et al., 1992) and *IGF2* mRNA expression (Drummond et al., 1992). Our panel of normal and malignant mesothelial cell lines showing variation in *WT1* mRNA expression was analyzed for *PDGFA* and *IGF2* mRNA levels as well. No clear relationship, reciprocal or otherwise, between *WT1* and *PDGFA* or *IGF2* expression was found in our cell lines. The fact that no clear correlation could be found in cells endogenously expressing these genes is in contrast with the repression seen in the forementioned studies. However, these data were obtained in cells upon transfection of *WT1* expression constructs together with *PDGFA* and *IGF2* reporter constructs, whereas we looked at endogenous expression levels in a panel of cell lines. It may also be that in our mesothelial cell lines other factors are also involved in regulating *PDGFA* and *IGF2* mRNA expression, thereby masking regulation by WT1. Alternatively, mutations in the WT1 binding sequences in the promoters of these genes may prohibit WT1 regulation. It remains to be determined, whether WT1 can regulate expression of these genes in a physiological context. Evidence for this may come from stable transfection of the *WT1* gene in Mero cell lines lacking *WT1* or knocking out the endogenous *WT1* gene expression in *WT1* expressing cell lines.

In summary, we have shown that *WT1* mRNA is consistently expressed in normal mesothelial cell lines and that there is no expression in a minority of malignant mesothelioma cell lines. No indications were found for chromosomal aberrations, deletions, rearrangements, functionally inactivating missense or nonsense mutations, or an aberrant alternative splicing pattern in these cell lines. The *WT1* expression level does not seem to correlate with the mesenchymal or epithelial morphology of the various cell lines in vitro. No inverse correlation between *WT1* and *PDGFA* or *IGF2* mRNA expression was seen in our panel of mesothelial cell lines, which endogenously express these genes.

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REFERENCES

Armstrong JF, Pritchard-Jones K, Bickmore WA, Hastie ND, Bard JBL (1992) The expression of the Wilms' tumour gene, *WT1*, in the developing mammalian embryo. Mech Dev 40:85–97.

Austin MB, Fechner RE, Roggli VL (1986) Pleural malignant mesothelioma following Wilms' tumor. Am J Clin Pathol 86:227–230.

Benham FJ, Hodgkinson S, Davies KE (1984) A glyceraldehyde-3-phosphate dehydrogenase pseudogene on the short arm of the human X-chromosome defines a multigene family. EMBO J 3:2635–2640.

Betsholtz C, Johnsson A, Heldin C-H, Westermark B, Lind P, Urdea MS, Eddy R, Shows TB, Philpott K, Mellor AL, Knott TJ, Scott J (1986) cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumor cell lines. Nature 320:695–699.

Bickmore WA, Oghene K, Little MH, Seawright A, van Heyningen V, Hastie ND (1992) Modulation of DNA binding specificity by alternative splicing of the Wilms' tumour *wt1* gene transcript. Science 257:235–237.

Brenner B, Wildhardt G, Schneider S, Royer-Pokora B (1992) RNA polymerase chain reaction detects different levels of four alternatively spliced *WT1* transcripts in Wilms' tumors. Oncogene 7:1431–1433.

Brown KW, Watson JE, Poirier V, Mott MG, Berry PJ, Maitland NJ (1992) Inactivation of the remaining allele of the *WT1* gene in a Wilms' tumour from a WAGR patient. Oncogene 7:763–768.

Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, Rose EA, Kral A, Yeger H, Lewis W, Jones C, Housman DE (1990) Isolation and characterization of a zinc-finger polypeptide gene at the human chromosome Wilms' tumor locus. Cell 60:509–520.

Connell ND, Rheinwald JG (1983) Regulation of the cytoskeleton in mesothelial cells: Reversible loss of keratin and increase in vimentin during rapid growth in culture. Cell 34:245–253.

Cotton RG, Rodrigues NR, Campbell RD (1988) Reactivity of cytosine and thymine in single-base-pair mismatches with hydroxylamine and osmium tetroxide and its application to the study of mutations. Proc Natl Acad Sci USA 85:4397–4401.

Cowell JK, Wadey RB, Haber DA, Call KM, Housman DE, Pritchard J (1991) Structural rearrangements of the *WT1* gene in Wilms' tumour cells. Oncogene 6:595–599.

Drummond IA, Madden SL, Rohwer-Nutter P, Bell GI, Sukhatme VP, Rauscher FJ III (1992) Repression of the insulin-like growth factor II gene by the Wilms' tumor suppressor WT1. Science 257:674–678.

Gashler AL, Bonthron DT, Madden SL, Rauscher FJ III, Collins T, Sukhatme VP (1992) Human platelet-derived growth factor A-chain is transcriptionally repressed by the Wilms' tumor suppressor WT1. Proc Natl Acad Sci USA 89:10984–10988.

Gessler M, Poustka A, Cavenee W, Neve RL, Orkin SH, Bruns GAP (1990) Homozygous deletion in Wilms' tumours of a zinc-finger gene identified by chromosome jumping. Nature 343:774–778.

Haber DA, Buckler AJ, Glaser T, Call KM, Pelletier J, Sohn RL, Douglass EC, Housman DE (1990) An internal deletion within an 11p13 zinc-finger gene contributes to the development of Wilms' tumor. Cell 61:1257–1269.

Haber DA, Sohn RL, Buckler AJ, Pelletier J, Call KM, Housman DE (1991) Alternative splicing and genomic structure of the Wilms' tumor gene *WT1*. Proc Natl Acad Sci USA 88:9618–9622.

Hagemeijer A, Versnel MA, van Drunen E, Moret M, Bouts MJ, van der Kwast ThH, Hoogsteden HC (1990) Cytogenetic analysis of malignant mesothelioma. Cancer Genet Cytogenet 47:1–28.

Hanson IM, Seawright A, Hardman K, Hodgson S, Zaletayev D,

Fekete G, van Heyningen V (1993) *PAX6* mutations in aniridia. Hum Mol Genet 2:915–920.
Huff V, Miwa H, Haber DA, Call KM, Housman DE, Strong L, Saunders GF (1991) Evidence for *WT1* as a Wilms' tumor (WT) gene: Intragenic germinal deletion in bilateral WT. Am J Hum Genet 48:997–1003.
Jansen M, van Schaik FMA, van Tol H, van den Brande JL, Sussenbach JS (1985) Nucleotide sequences of cDNAs encoding precursors of human insulin-like growth factor-II (IGF-II) and an IGF-II variant. FEBS Lett 179:243–246.
Kreidberg JA, Sariola H, Loring JM, Maeda M, Pelletier J, Housman D, Jaenisch R (1993) WT-1 is required for early kidney development. Cell 74:679–691.
Langerak AW, Dirks RPH, Versnel MA (1992) Splicing of the platelet-derived growth factor A-chain mRNA in human malignant mesothelioma cell lines and regulation of its expression. Eur J Biochem 208:589–596.
Little MH, Prosser J, Condie A, Smith PJ, van Heyningen V, Hastie ND (1992) Zinc finger point mutations within the *WT1* gene in Wilms' tumor patients. Proc Natl Acad Sci USA 89:4791–4795.
Madden SL, Cook DM, Morris JF, Gashler A, Sukhatme VP, Rauscher FJ III (1991) Transcriptional repression mediated by the WT1 Wilms' tumor gene product. Science 253:1550–1553.
Maheswaran S, Park S, Bernard A, Morris JF, Rauscher FJ III, Hill DE, Haber DA (1993) Physical and functional interactions between WT1 and p53 proteins. Proc Natl Acad Sci USA 90:5100–5104.
Papadaki L, Beilby JOW (1971) The fine structure of the surface epithelium of the human ovary. J Cell Sci 8:445–465.
Park S, Tomlinson G, Nisen P, Haber DA (1993a) Altered transactivational properties of a mutated *WT1* gene product in a WAGR-associated Wilms' tumor. Cancer Res 53:4757–4760.
Park S, Schalling M, Bernard A, Maheswaran S, Shipley GC, Roberts D, Fletcher J, Shipman R, Rheinwald J, Demetri G, Griffin J, Minden M, Housman DE, Haber DA (1993b) The Wilms' tumor gene *WT1* is expressed in murine mesoderm-derived tissues and mutated in a human mesothelioma. Nat Genet 4:415–420.
Pelletier J, Schalling M, Buckler AJ, Rogers A, Haber DA, Housman DE (1991) Expression of the Wilms' tumour gene *WT1* in the murine urogenital system. Genes Dev 5:1345–1356.
Pritchard-Jones K, Fleming S, Davidson D, Bickmore W, Porteous D, Gosden C, Bard J, Buckler A, Pelletier J, Housman D, van Heyningen V, Hastie ND (1990) The candidate Wilms' tumour gene is involved in genitourinary development. Nature 346:194–197.
Prosser J, Thompson AM, Cranston G, Evans HJ (1990) Evidence that p53 behaves as a tumour suppressor gene in sporadic breast tumours. Oncogene 5:1573–1579.
Rauscher FJ III, Morris JF, Tournay OE, Cook DM, Curran T (1990) Binding of the Wilms' tumor locus zinc-finger protein to the EGR-1 consensus sequence. Science 250:1259–1262.
Riccardi VM, Sujanski E, Smith AC, Francke U (1978) Chromosomal imbalance in the aniridia-Wilms' tumor association: 11p interstitial deletion. Pediatrics 61:604–610.
Tadokoro K, Fujii H, Ohshima A, Kakizawa Y, Shimizu K, Sakai A, Sumiyoshi K, Inoue T, Hayashi Y, Yamada M (1992) Intragenic homozygous deletion of the *WT1* gene in Wilms' tumor. Oncogene 7:1215–1221.
van Heyningen V, Boyd PA, Seawright A, Fletcher JM, Fantes JA, Buckton KE, Spowart G, Porteous DJ, Hill RE, Newton MS, Hastie ND (1985) Molecular analysis of chromosome 11 deletions in aniridia-Wilms' tumour syndrome. Proc Natl Acad Sci USA 82:8592–8596.
Versnel MA, Hagemeijer A, Bouts MJ, van der Kwast ThH, Hoogsteden HC (1988) Expression of c-*sis* (PDGF B-chain) and PDGF A-chain genes in ten human malignant mesothelioma cell lines derived from primary and metastatic tumours. Oncogene 2:601–605.
Versnel MA, Bouts MJ, Hoogsteden HC, van der Kwast ThH, Delahaye M, Hagemeijer A (1989) Establishment of human malignant mesothelioma cell lines. Int J Cancer 44:256–260.
Versnel MA, Claesson-Welsh L, Hammacher A, Bouts MJ, van der Kwast ThH, Eriksson A, Willemsen R, Weima SM, Hoogsteden HC, Hagemeijer A, Heldin C-H (1991) Human malignant mesothelioma cell lines express PDGF β-receptors, whereas cultured normal mesothelial cells express predominantly PDGF α-receptors. Oncogene 6:2005–2011.
Versnel MA, Langerak AW, van der Kwast ThH, Hoogsteden HC, Hagemeijer A (1993) Expression of PDGF chains and PDGF receptors in normal and malignant mesothelial cell lines. Eur Respir Rev 3:186–188.
Versnel MA, Haarbrink M, Langerak AW, de Laat PAJM, Hagemeijer A, van den Berg L, Schrier PI (1994) Human ovarian tumours from epithelial origin express PDGF in vitro and in vivo. Cancer Genet Cytogenet 73:60–64.
Wagner JC, Sleggs CA, Marchand P (1960) Diffuse pleural mesothelioma and asbestos exposure in the North-Western Cape Province. Br J Ind Med 17:260–270.
Wang ZY, Madden SL, Deuel TF, Rauscher FJ III (1992) The Wilms' tumor gene product *WT1* represses transcription of the platelet-derived growth factor A-chain gene. J Biol Chem 267:21999–22002.
Wang ZY, Qiu QQ, Deuel TF (1993) The Wilms' tumor gene product *WT1* activates or suppresses transcription through separate functional domains. J Biol Chem 268:9172–9175.
Werner H, Re GG, Drummond IA, Sukhatme VP, Rauscher FJ III, Sens DA, Garvin AJ, LeRoith D, Roberts C (1993) Increased expression of the insulin-like growth factor I receptor gene, *IGF1R*, in Wilms' tumor is correlated with modulation of *IGF1R* promoter activity by the *WT1* Wilms' tumor gene product. Proc Natl Acad Sci USA 90:5828–5832.
Winship PR (1989) An improved method for directly sequencing PCR amplified material using dimethyl sulphoxide. Nucleic Acids Res 17:1266.

6

GENERAL DISCUSSION

CHAPTER 6

GENERAL DISCUSSION

Human malignant mesothelioma is a tumour of mesodermal origin. The incidence of malignant mesothelioma is strongly associated with exposure to asbestos fibers. Continued growth, the hallmark of transformed cells, is known to result from uncontrolled expression and action of growth-regulating molecules. One of the, for this reason, most extensively studied growth factors in mesothelioma, is platelet-derived growth factor (PDGF) (1-6). PDGF has been shown to be mitogenic for mesenchymal cells, i.e. for those mesenchymal cells that express the appropriate receptors for PDGF. The studies described in this thesis concern the expression of PDGF chains and receptors and their regulation in malignant mesothelioma cells.

Expression pattern of PDGF chains and receptors in mesothelial cells *in vitro* and *in vivo*

Human malignant mesothelioma cell lines were found to express PDGF A- and B-chain as well as PDGF ß-receptor mRNA, whereas cultured normal mesothelial cells demonstrated low expression levels of PDGF A-chain mRNA, PDGF α-receptor mRNA and only occasionally ß-receptor mRNA, but no PDGF B-chain transcripts (1-3; Chapters 3.1 and 3.2). Using other assays, more sensitive than Northern blotting, to detect RNA, PDGF α-receptors could also be identified in malignant mesothelioma cells (5; Chapter 4.3). The PDGF chain and receptor mRNA expression patterns of normal and malignant mesothelial cells were found to be largely comparable to their respective protein patterns (Chapter 3.2). Moreover, expression in normal and malignant mesothelial cells *in vivo* (i.e. in primary tissues and in effusions) turned out to be quite similar to that observed *in vitro*. In murine mesothelioma cell lines a pattern comparable to that in human cell lines was seen (6), whereas no PDGF A- and/or B-chain mRNA was observed in rat mesothelioma cells (7).

Based on PDGF chain and receptor expression data and on the receptor subunit model as suggested by Seifert et al. (8), several possible interactions between PDGF chains and receptors could in theory play a role in mesothelial cell lines. The observation that mesothelioma cells *in vitro* and *in vivo* express both PDGF and PDGF ß-receptors, supports the hypothesis that PDGF (probably PDGF B-chain dimers) may be involved in an autocrine loop in mesothelioma cells. Other interactions between PDGF isoforms and receptors (e.g. between PDGF A-chain dimers and PDGF α-receptors) may play a role in uncontrolled growth stimulation as well. It has been shown that PDGF α- and ß-receptors can activate common signalling pathways (9), but it is also known that these receptor types possess different substrate specificities (9,10). The actual contribution of all PDGF chain/receptor interactions to growth stimulation will therefore have to be tested in further experiments. Special attention should be paid to analyzing the functional importance of a potential interaction between PDGF-BB and the PDGF ß-

receptor dimer. To this end, the autophosphorylation status of PDGF ß-receptors should be analyzed in the mesothelioma cells. Furthermore, interference in the potential autocrine loop by introduction of truncated PDGF ß-receptors or receptor antagonists should reveal its importance for the pathogenesis of malignant mesothelioma. Comparable strategies have been applied successfully in malignancies derived from other cell types. Introduction of dominant-negative PDGF mutants and truncated PDGF ß-receptors in astrocytoma and glioma cells, respectively, resulted in (partial) reversal of the transformed phenotype by disruption of PDGF-induced growth-stimulating loops (11,12). In other cell types neutralizing antibodies directed against the PDGF α-receptor (13) or against PDGF (14) have also been used to inhibit PDGF autocrine growth stimulation. However, the antibody approach does not seem suitable for mesothelioma cells, because the PDGF receptor is probably activated intracellularly, as no clear PDGF-like activity could be identified in the supernatant (unpublished results). Small compounds called tyrphostins have recently been described as selective tyrosine kinase blockers of PDGF and stem cell factor receptors (15). Antisense PDGF B-chain oligonucleotides may be another tool to inhibit PDGF-induced growth stimulation (16), but success strongly depends on the stability of the oligonucleotides. In this respect the use of other small RNA molecules, ribozymes, that cleave their target RNA, is more promising. It has been shown recently that addition of a ribozyme directed against the PDGF B-chain modulated growth of a mesothelioma cell line (17). It would also be interesting to check whether a recently identified candidate tumour suppressor gene that is homologous to the extracellular domain of the PDGF β-receptor gene, is inactivated in mesothelioma cells, as it is hypothesized that functional loss of this protein, that might act as an antagonist of growth factor/receptor interactions, may contribute to the carcinogenesis of certain cell types (18).

In view of the potential role PDGF plays in malignant mesothelioma, regulation of PDGF chain and receptor gene expression in mesothelial cells is an important topic to study. Our normal and malignant mesothelial cell lines provide a good model system to obtain detailed knowledge on these processes and on the potential mechanisms that may explain these differential expression patterns. Data resulting from these studies may also be relevant for other cell types in which increased PDGF chain and receptor expression is seen, although they should be tested in that particular cell type as well.

Regulation of PDGF A-chain expression in mesothelial cells

As normal and malignant mesothelial cells are cultured under slightly different conditions (i.e. extra addition of EGF and HC to the culture medium of normal mesothelial cells), we first tested whether these conditions could explain the differential expression pattern. Collectively, the results suggested that this is not the case. In summary, EGF and HC did not significantly affect PDGF A- and B-chain mRNA levels, whereas the PDGF α- and ß-receptor messenger levels were slighlty affected (Chapter 4.1, 4.3 and data not shown). In addition to this, both normal and malignant mesothelial cells did not demonstrate detectable expression of the otherwise undetectable receptor subtype under these conditions, using Northern blot analysis. Together, these data support the existence of intrinsic differences in PDGF chain and receptor expression between normal and malignant mesothelial cells.

Despite the many structural and numerical chromosomal abnormalities of malignant mesothelioma cells, no consistent aberration was found that could account for the observed difference in PDGF A-chain or PDGF B-chain mRNA expression (Chapter 4.1 and 4.2). Amplifications, rearrangements or deletions of the genes encoding these growth factor chains could not be detected either. Nuclear run off and CHX-stimulation experiments suggested increased transcription to be the cause of the elevated PDGF A-chain (Chapter 4.1) as well as PDGF B-chain (Chapter 4.2) mRNA levels in malignant mesothelioma cell lines as compared to their normal counterparts. PDGF A-chain mRNA half-lives were similar in normal and malignant mesothelial cells (data not shown).

Activation of PDGF A-chain transcription was studied in many reports. The promoter region was found to contain the start site for the three PDGF A-chain transcripts (19,20). The PDGF A-chain promoter was found to consist of a TATA box and a GC-rich stretch of DNA (-150/-33) which contains roughly 80% of promoter activity (21). The region was further found to contain consensus DNA binding sequences for Sp1-like factors (22). In a region downstream of the transcription start site (+50/+67) a (novel) binding site for transcription factor Egr-1 was observed (23). Transcriptional activity of the PDGF A-chain gene also appeared to be inhibited by site-specific methylation of CpG sites in the highly GC-rich promoter (24). Future experiments should clarify whether demethylation of the promoter region or increased binding of Sp1- or Egr-1-like factors causes increased PDGF A-chain transcription in malignant mesothelioma cells as well. Alternatively, negative regulating regions as identified in the first exon or first intron (25,26), or other cell type-specific regulating regions may play a role in these cells. Next to transcription regulation, the PDGF A-chain possesses an additional way to regulate its mRNA expression, that is through alternative splicing. Our studies revealed that both normal and malignant mesothelial cell lines were mainly found to produce transcripts without the exon 6-derived sequence that has been implicated in ECM binding and cell retention (Chapter 4.1).

Regulation of PDGF B-chain expression in mesothelial cells

Activation of PDGF B-chain transcription in malignant mesothelioma cell lines was studied in detail (Chapter 4.2). Binding of one or more factors to the region around nucleotide -61 and to the region around the transcription start site was observed by *in vivo* footprint assays. The concomitant decrease in reporter gene activity upon deletion of this region strongly suggested that it contains (part of) an activator (Chapter 4.2). Although the non-expressing normal mesothelial cells also demonstrated a small decrease in reporter activity, no binding was seen, suggesting that actual binding to the endogenous B-chain promoter is hindered by other mechanisms. In other cell types a minimal PDGF B-chain promoter region was found to extend to residue -72 (27), with a small region (-64/-45) being especially important in this respect (28). By a combination of reporter gene analysis and gel mobility shift assays or *in vivo* footprinting analysis other investigators identified regulatory sequences in this region as well. A CCACCCAC element (-61/-54) was identified as the binding site for a strong transcriptional activator, possibly Sp1, in TPA-treated K562 cells and osteosarcoma cells (29-31), whereas a TCTC sequence (-64/-61), was identified as (part of) a weak transcriptional activator in untreated K562 cells and prostate carcinoma (PC3) cells (31). The factor that may bind

to this TCTC sequence has not been identified yet. Dirks et al. (32) showed that there was no transcription regulating effect when the promoter was confined to 112 bp upstream of the transcription start, which is similar to what we have seen, but different from the clear decreasing effect that was seen from the region -372/-343 by Pech et al. (27).

Analysis of many cell types has revealed that activation of PDGF B-chain transcription is a very complex process involving both positive and negative regulating elements located upstream, downstream and within the transcription unit. Moreover, many of these sequences were found to act in a cell type-specific manner (32,33). Data from reporter assays in malignant mesothelioma cells are suggestive of the involvement of a DNaseI hypersensitive (DH) region at -9.9 kb of the PDGF B-chain transcription start in enhancing PDGF B-chain mRNA expression in these cells (Chapter 4.2). A DH site at -9.9 kb, and also one at -8.6 kb, showed strong enhancer activity in TPA-treated K562 cells as well. In HeLa and PC3 cells the individual sites also showed enhancer activity, but when combined in a larger fragment this activity was almost completely lost (34). The latter is also partly the case in mesothelioma cells, so it remains to be determined to what extent the -9.9 kb region contributes to elevated PDGF B-chain expression in the context of the genome. Future experiments should clarify the identity of the factor(s) that bind to this -9.9 kb region, and also to the sequence around nucleotide -61, in malignant mesothelioma cell lines.

Regulation of PDGF α- and ß-receptor expression in mesothelial cells

As for the PDGF chains, no consistent chromosomal or gene aberrations were observed that could account for the modified expression of PDGF receptors. However, unlike the situation for the PDGF chains, the differences in PDGF α- and ß-receptor steady-state mRNA levels between normal and malignant mesothelial cells could not simply be explained by differences in transcription only (Chapter 4.3). The lack of clear PDGF ß-receptor expression in normal mesothelial cells seemed to be determined at the transcriptional level. However, PDGF α-receptor mRNA expression was mainly regulated at the post-transcriptional level, since mesothelioma cells were found capable of producing α-receptor transcripts as well, using more sensitive assays like nuclear run off and RT-PCR analysis. The inability to detect α-receptor mRNA by Northern blot analysis is apparently due to different degradation mechanisms between normal and malignant mesothelial cells. The α-receptor was found to be rather stable in the former and may be rapidly degraded in the latter (Chapter 4.3). Although several studies have been published identifying factors involved in regulation of PDGF receptor mRNA levels, only a limited amount of data is available on involvement of such factors in PDGF α-receptor transcription. Molecular cloning of the murine PDGF α-receptor promoter revealed a 93 bp region that conferred tissue-specific PDGF α-receptor expression (35). The human PDGF α-receptor promoter was found to be slightly larger (36). It remains to be determined whether this region is able to drive PDGF α-receptor transcription in mesothelial cells as well. In human teratocarcinoma cells alternative promoter use was observed, which, in combination with alternative splicing, resulted in novel developmentally regulated PDGF α-receptor transcripts (37). PDGF α-receptor transcripts of a similar size were not seen in the mesothelial cells. Thus far nothing has

been published on activation of human PDGF ß-receptor transcription or on the molecular cloning of its promoter. A 1.9 kb promoter fragment of the murine PDGF β-receptor gene has only recently been cloned and analyzed by functional promoter assays (38). Future studies will possibly reveal (additional) general and cell type-specific enhancing and repressive elements of both PDGF α and ß-receptor genes. The mesothelial cell lines may be used for such studies.

As already mentioned, many factors are capable of modulating PDGF receptor expression. Stimulation of malignant mesothelioma cells with TGF-ß1, which is one of the most extensively studied regulators, resulted in increased PDGF ß-receptor mRNA levels in 2 out of 6 cell lines, whereas no clear effect was seen in the remainder (Chapter 4.3). As mesothelial cells produce TGF-ß themselves, the cells themselves may thus in some cases be capable of modulating the PDGF autocrine loop in a positive way via TGF-ß1. In fibroblasts, vascular smooth muscle cells and mesangial cells upregulation of PDGF ß-receptor mRNA and protein expression by TGF-ß was shown to be accompanied by increased PDGF B-chain mRNA expression (40-42). However, in our cell lines no effect on PDGF B-chain mRNA levels was seen (data not shown). TGF-ß1 stimulation of normal mesothelial cells resulted in decreased PDGF α-receptor levels, which was also seen by others in cultured human fibroblasts (43) and smooth muscle cells (44).

WT1 as a transcriptional regulator in mesothelial cells?

The WT1 gene product has been shown to have DNA binding and transcription-regulating capacity. In transient transfection studies a.o. the PDGF A-chain, IGF2, and IGFIR genes have been identified as potential target genes for regulation by WT1 (45-48). The fact that in these studies WT1 was found to repress reporter constructs containing the promoter regions of these genes, has led to the hypothesis that reduction or absence of WT1 expression results in a higher expression of important growth factor (receptor) genes such as PDGF A-chain and IGF2, thereby contributing to tumorigenesis. We therefore studied WT1 for its putative role as regulator of PDGF A-chain gene expression in mesothelial cell lines as well. However, when analyzing the WT1 and PDGF A-chain mRNA levels in our entire panel of malignant mesothelioma cell lines, we could not find any clear correlation that was suggestive of repression (nor of activation) by WT1 (Chapter 5.2). The same holds true for the WT1 and IGF2 levels. This is different from the earlier mentioned transfection studies. However, these transient studies only provide information about the ability of WT1 to bind and activate part of the target promoter in a reporter construct, but not about actual regulation of expression of the endogenous target gene in question. The discrepancy may thus be explained by the nature of the analysis, since in our studies we did analyze endogenous expression levels of target genes. Alternatively, it may be that other (unknown) factors, also regulating PDGF A-chain and/or IGF2 mRNA expression, mask the otherwise clear relationship between WT1 and PDGF A-chain/IGF2. The observation that WT1 acts as a repressor in the presence of functional p53 and as an activator in the absence of p53, is important in this respect (49), although a physiological interaction between WT1 and p53 *in vivo* remains to be shown. Rodeck et al. (50) hypothesized that WT1 action is indeed influenced by the presence of mutant p53 in a few of their melanoma cell lines.

Preliminary results indicate that one of our malignant mesothelioma cell lines does not seem to express p53 mRNA. Most interestingly, this is cell line Mero-25, which has the highest WT1 mRNA expression level. If the beforementioned idea turns out to be correct, WT1 may thus play a role as a strong activator rather than an repressor in this particular cell line.

To solve the question of WT1 transcription regulating capacity, further experiments should be performed using the mesothelioma cell lines as a model system. In these experiments the relationship between WT1 and PDGF A-chain or other target genes, will have to be studied after modulation of WT1 protein expression levels by means of introducing WT1 gene constructs or by functional inactivation of the WT1 gene in WT1-expressing malignant mesothelioma cell lines.

The possible role of WT1 and other tumour suppressor genes in mesothelial carcinogenesis

Expression of the WT1 tumour suppressor gene can be observed in a limited set of tissues that experience in common a mesenchyme to epithelial transition during their development (51-53). WT1 expression was also observed in the mesothelium, which is another tissue known to have undergone such a transition (53-55; Chapter 5.2). Since in Wilms tumours WT1 is one of the loci involved in carcinogenesis, being mutated or inactivated in roughly 20% of cases, its putative contribution to mesothelial carcinogenesis was also studied. We found lack of WT1 mRNA expression in 4 out of 13 mesothelioma cell lines (Chapter 5.2), whereas others showed consistent, though highly variable, expression in their cell lines (55). In all cases no differences in the WT1 splicing pattern were observed (55; Chapter 5.2). Furthermore, WT1 point mutations that are frequently observed in Wilms tumours occurring in Denys-Drash syndrome patients (56-58), could not be found in the analyzed WT1 exons in several (pleural) malignant mesotheliomas and mesothelioma cell lines (54,59; Chapter 5.2). It may still be that mutations in the 5' most coding region of exon 1 have remained undetected. Recently, somatic and germline mutations have been observed in a few Wilms tumour patients in this part of the WT1 gene, that contains many potential hotspot motifs for deletions and insertions (60). In a single case of multicystic peritoneal mesothelioma a point mutation was found in the WT1 transregulation domain (54). However, this type of mesothelioma is non-asbestos related and is considered a non-neoplastic mesothelial proliferation rather than a malignancy (61). Together these data suggest that the role of WT1 is limited to only a subset of pleural mesotheliomas. This aspect should be further addressed by immunohistochemical and RNA *in situ* hybridization analysis of primary mesothelioma samples. In a recent report WT1 protein expression could indeed be detected immunohistochemically in the majority of malignant mesothelioma tumour samples studied (62). Cases of non-asbestos related (childhood) mesothelioma may be worth analyzing as well, given the fact that malignant mesothelioma was mentioned as a second-risk tumour in a small number of patients that had recovered from Wilms tumour (63).

The RB and p53 tumour suppressor genes were studied in malignant mesothelioma as well. Lack of RB mRNA and/or protein were not observed in several human mesothelioma cell lines (64,65), but not all samples were screened for small (point)

mutations. p53 was detected immunohistochemically in a considerable proportion (25-70%) of mesotheliomas (66-69). This detectable expression was interpreted as overexpression of a mutant form of p53. However, the correlation between immunocytochemical detection and mutation of p53 is still a point of discussion (70,71). Actual aberrant (point mutated) p53 expression or lack of p53 expression was seen in only a minority of malignant mesotheliomas (72,73; our unpublished results). Inactivation of either RB or p53 therefore does not seem to be a common event in malignant mesothelioma cells. The occurrence of mutations and (micro)deletions in the neurofibromatosis type 2 (NF2) gene, implicates this gene in the oncogenesis of at least a subset of mesotheliomas (74).

Cytogenetic analysis of human malignant mesothelioma tumour samples and/or cell line material has revealed a complex heterogeneous pattern of chromosomal abnormalities, involving non-random loss of chromosomes 1p (1p21-22), 3p (3p21), 4, 6q (6q15-21), 9p (9p21-22), 22 and gain of chromosomes 5 and 7 at high frequencies (75-83). Chromosome regions that are lost in the majority of mesotheliomas are putative sites of (unknown) tumour suppressor genes, involved in mesothelial carcinogenesis. Recently, two potential tumour suppressor genes were mapped to the 9p21 region, which is frequently deleted in malignant mesothelioma as well. One of these genes, called MTS1, encodes a 16 kDa protein (p16) that acts as an inhibitor of cyclin-dependent kinase 4 (CDK4) and is frequently deleted in many tumour types (84,85). MTS2 (the p15 gene) is rather homologous to MTS1 and appears to encode a 15 kDa CDK4/CDK6 inhibitor that seems to be involved in TGF-ß-mediated cell cycle arrest (86). Analysis of the p16 gene revealed that this gene is homozygously deleted in a high proportion of malignant mesothelioma cell lines as well, and that homozygous deletions can also be identified in primary tumour samples, albeit at a lower frequency (87; JB Prins, pers. comm.).

The role of PDGF and other growth factors in mesothelial carcinogenesis

Cancer is considered a multi-step process in which both (proto)-oncogenes and tumour suppressor genes are involved. The importance of the co-expression of PDGF B-chains and ß-receptors for autocrine growth stimulation of mesothelioma cells has already been highlighted and discussed in the previous parts of this chapter. Furthermore, the PDGF produced by mesothelioma cells may also exert a paracrine effect. A situation may thus be envisaged that is comparable to other tumour types where secreted PDGF supports tumour growth by stimulating PDGF receptor-containing stromal cells (endothelial cells, fibroblasts). It can be hypothesized that activation of PDGF α-receptors on non-transformed mesothelial cells and fibroblasts by mesothelioma-secreted PDGF-AA may support stromal growth in mesotheliomas. However, despite the possible importance of PDGF-induced autocrine and paracrine growth-stimulating signals, PDGF will certainly not be the only factor involved in growth stimulation and possibly transformation of mesothelioma cells. In a recent publication Kim et al. (88) showed that transformation by PDGF-AA or -BB isoforms requires a permissive (or inductive) genotype in a multi-step transformation process, underscoring the idea that autocrine growth stimulation by PDGF may be required but will not be sufficient for transformation. PDGF is an example of a growth factor that belongs to the

category of so-called competence factors, that are involved in rendering cells competent to progress through the G_1 phase of the cell cycle. For this progression, other factors are needed. Examples of such progression factors are IGF1 and EGF. There are a few reports providing indirect evidence that IGF1 may play a role in the pathogenesis of mesothelioma as well. Firstly, simultaneous expression of IGF1 and IGF1 receptor was reported in mesothelioma cells (89), Secondly, high levels of IGF1 protein were observed in pleural fluids of mesothelioma patients (90). Evidence for an autocrine role for TGF-α (acting via the EGF receptor) in mesothelioma cells and in asbestos-transformed mesothelial cells has been presented as well (91,92).

Growth factors like PDGF-BB (93,94) and also CSF1 (95) have been found to increase cyclin D1 mRNA levels in fibroblasts. It remains to be shown that the competence factor PDGF increases cyclin D1 levels in malignant mesothelioma cells as well. Based on this assumption, however, it is tempting to speculate that in the mesothelioma cells a PDGF-induced increase in cyclin D1 product may act in concert with a decreased sequestration of CDK4 by its inhibitor p16 (see before). This combination may lead the cell through its restriction point in G_1 of the cycle. Although this deregulation of the cell cycle provides a nice mechanism for uncontrolled growth of mesothelioma cells, it may prove too simple, as the cell may possess other inhibiting factors that can replace the ones that are inactivated. Moreover, as transition trough G_1 depends on the activity of other cyclin/CDK complexes as well, the mere continuous cyclin D1/CDK4 activity may not be sufficient for sustained growth, as deregulated growth-stimulating signals acting via other cyclin/CDK complexes may contribute as well. Experiments will thus have to be performed to evaluate the contribution of these complexes to the transformation of malignant mesothelioma cells.

Possible diagnostic and therapeutic implications

Simultaneous expression of PDGF B-chain and PDGF ß-receptor gene products is not specific for mesothelial tumours, but seems to contribute to the transformed character of e.g. lung epithelial tumours as well (96). Therefore, expression of these molecules is not useful as a marker to discriminate between mesothelioma and lung adenocarcinoma, which constitutes one of the main difficulties in the diagnosis of malignant mesothelioma. Furthermore, although in effusions normal and malignant mesothelial cells show a differential PDGF expression pattern, these expressions are too faint to be used as markers for reactive or transformed mesothelial cells. Moreover, this is further hampered by the lack of antibodies that can discriminate between PDGF-AA and -BB. Recently appropriate antibodies have become available for WT1 immunocytochemical or -histochemical detection. In view of its tissue-specific expression, positive identification of WT1 proteins may prove useful, especially as a diagnostic tool to discriminate between malignant pleural mesotheliomas and non-WT1-expressing adenocarcinomas of the lung, as has recently been suggested by Amin et al. (62) as well. However, a negative result will still be inconclusive, as this may also result from loss of WT1 protein expression in malignant mesothelioma cells. Analysis of WT1 protein expression does not allow for discrimination between neoplastic mesothelial cells and untransformed mesothelial cells (Chapter 5.2), which forms another difficulty in the diagnosis of malignant mesothelioma.

Mesothelioma is a malignancy that seems rather refractory to all kinds of conventional therapy. One kind of therapy that is being considered for future applications in several cancer types, is somatic gene therapy. The use of viral vectors is one method for insertion of genetic material into cells of interest, but other (more reliable) methods are under study as well. Recently, a study has been published in which genetic material was successfully transferred in human malignant mesothelioma cells *in vitro* and *in vivo*, using adenoviral vectors (97). Successful gene-based therapy also requires an appropriate target. In this respect one could think of wild-type forms of inactive tumour suppressor genes or of antisense forms of relevant oncogenes. The results described in this thesis together with studies of others have further stressed PDGF and/or PDGF receptors as potential targets for gene therapy in (a subset of) malignant mesotheliomas. However, much is to be learned before this kind of therapy can be successfully applied in the clinic. Meanwhile, studies using malignant mesothelioma cell lines will be extremely helpful in evaluating the potential value of such strategies.

REFERENCES

1. Gerwin BI, Lechner JF, Reddel RR, Roberts AB, Robbins KC, Gabrielson EW, Harris CC. Comparison of production of transforming growth factor-β and platelet-derived growth factor by normal human mesothelial cells and mesothelioma cell lines. Cancer Res. 47: 6180-6184, 1987.
2. Versnel MA, Hagemeijer A, Bouts MJ, Van der Kwast ThH, Hoogsteden HC. Expression of c-sis (PDGF B-chain) and PDGF A-chain genes in ten human malignant mesothelioma cell lines derived from primary and metastatic tumors. Oncogene 2: 601-605, 1988.
3. Versnel MA, Claesson-Welsh L, Hammacher A, Bouts MJ, Van der Kwast ThH, Eriksson A, Willemsen R, Welma SM, Hoogsteden HC, Hagemeijer A, Heldin C-H. Human malignant mesothelioma cell lines express PDGF β-receptors whereas cultured normal mesothelial cells express predominantly PDGF α-receptors. Oncogene 6: 2005-2011, 1991.
4. Ramael M, Buysse C, Van den Bossche J, Segers K, Van Marck E. Immunoreactivity for the β chain of the platelet-derived growth factor receptor in malignant mesothelioma and non-neoplastic mesothelium. J. Pathol. 167: 1-4, 1992.
5. Garlepp MJ, Christmas TI, Manning LS, Mutsaers SE, Dench J, Leong C, Robinson BWS. The role of platelet-derived growth factor in the growth of human malignant mesothelioma. Eur. Resp. Rev. 3: 189-191, 1993.
6. Garlepp MJ, Christmas TI, Mutsaers SE, Manning LS, Davis MR, Robinson BWS. Platelet-derived growth factor as an autocrine factor in murine malignant mesothelioma. Eur. Resp. Rev. 3: 192-194, 1993.
7. Walker C, Bermudez E, Stewart W, Bonner J, Molloy CJ, Everitt J. Characterization of platelet-derived growth factor and platelet-derived growth factor receptor expression in asbestos-induced rat mesothelioma. Cancer Res. 52: 301-306, 1992.
8. Seifert RA, Hart CE, Phillips PE, Forstrom JW, Ross R, Murray MJ, Bowen-Pope DF. Two different subunits associate to create isoform-specific platelet-derived growth factor receptors. J. Biol. Chem. 264: 8771-8778, 1989.
9. Eriksson A, Siegbahn A, Westermark B, Heldin C-H, Claesson-Welsh L. PDGF α- and ß-receptors activate unique and common signal transduction pathways. EMBO J. 11: 543-550, 1992.
10. Heidaran MA, Beeler JF, Yu J-C, Ishibashi T, LaRochelle WJ, Pierce JH, Aaronson SA. Differences in substrate specificities of α and ß platelet-derived growth factor (PDGF) receptors. J. Biol. Chem. 268: 9287-9295, 1993.
11. Shamah SM, Stiles CD, Guha A. Dominant-negative mutants of platelet-derived growth factor revert the transformed phenotype of human astrocytoma cells. Mol. Cell. Biol. 13: 7203-7212, 1993.
12. Strawn LM, Mann E, Elliger SS, Chu LM, Germain LL, Niederfellner G, Ullrich A, Shawver LK. Inhibition of glioma cell growth by a truncated platelet-derived growth factor-ß receptor. J. Biol. Chem. 269: 21215-21222, 1994.

13. LaRochelle WJ, Jensen RA, Heidaran MA, May-Siroff M, Wang LM, Aaronson SA, Pierce JH. Inhibition of platelet-derived growth factor autocrine growth stimulation by a monoclonal antibody to the human α platelet-derived growth factor receptor. Cell Growth Differ. 4: 547-553, 1993.
14. Vassbotn F, Östman A, Langeland N, Holmsen H, Westermark B, Heldin C-H, Nistér M. Activated platelet-derived growth factor autocrine pathway drives the transformed phenotype of a human glioblastoma cell line. J. Cell. Physiol. 158: 381-389, 1994.
15. Kovalenko M, Gazit A, Böhmer A, Rorsman C, Rönnstrand L, Heldin C-H, Waltenberger J, Böhmer F-D, Levitzki A. Selective platelet-derived growth factor receptor kinase blockers reverse *sis*-transformation. Cancer Res. 54: 6106-6114, 1994.
16. Souza P, Sedlackova L, Kuliszewski M, Wang J, Liu J, Tseu I, Liu M, Tanswell AK, Post M. Antisense oligodeoxynucleotides targeting PDGF-B mRNA inhibit cell proliferation during embryonic rat lung development. Development 120: 2163-2173, 1994.
17. Dorai T, Kobayashi H, Holland JF, Ohnuma T. Modulation of platelet-derived growth factor-ß mRNA expression and cell growth in a human mesothelioma cell line by a hammerhead ribozyme. Mol. Pharmacol. 46: 437-444, 1994.
18. Fujiwara Y, Ohata H, Kuroki T, Koyama K, Tsuchiya E, Monden M, Nakamura Y. Isolation of a candidate tumor suppressor gene on chromosome 8p21.3-p22 that is homologous to an extracellular domain of the PDGF receptor beta gene. Oncogene 10: 891-895, 1995.
19. Bonthron DT, Morton CC, Orkin SH, Collins T. Platelet-derived growth factor A chain: Gene structure, chromosomal location, and basis for alternative mRNA splicing. Proc. Natl. Acad. Sci. USA 85: 1492-1496, 1988.
20. Takimoto Y, Wang ZY, Kobler K, Deuel TF. Promoter region of the human platelet-derived growth factor A-chain gene. Proc. Natl. Acad. Sci. USA 88: 1686-1690, 1991.
21. Lin XH, Wang ZY, Gu LJ, Deuel TF. Functional analysis of the human platelet-derived growth factor A-chain promoter region. J. Biol. Chem. 267: 25614-25619, 1992.
22. Kaetzel DM, Maul RS, Liu B, Bonthron D, Fenstermaker RA, Coyne DW. Platelet-derived growth factor A-chain gene transcription is mediated by positive and negative regulatory regions in the promoter. Biochem. J. 301: 321-327, 1994.
23. Wang ZY, Deuel TF. An S1 nuclease-sensitive homopurine/homopyrimidine domain in the PDGF A-chain promoter contains a novel binding site for the growth factor-inducible protein EGR-1. Biochem. Biophys. Res. Comm. 188: 433-439, 1992.
24. Lin XH, Guo C, Gu LJ, Deuel TF. Site-specific methylation inhibits transcriptional activity of platelet-derived growth factor A-chain promoter. J. Biol. Chem. 268: 17334-17340, 1993.
25. Takimoto Y, Kuramoto A. Gene regulation by the 5'-untranslated region of the platelet-derived growth factor A-chain. Bioch. Biophys. Acta 1222: 511-514, 1994.
26. Wang ZY, Masaharu N, Qiu QQ, Deuel TF. An S1 nuclease-sensitive region in the first intron of human platelet-derived growth factor A-chain gene contains a negatively acting cell type-specific regulatory element. Nucleic Acids Res. 22: 457-464, 1994.
27. Pech M, Rao CD, Robbins KC, Aaronson SA. Functional identification of regulatory elements within the promoter region of platelet-derived growth factor 2. Mol. Cell. Biol. 9: 396-405, 1989.
28. Jin H-M, Brady ML, Fahl WE. Identification and characterization of an essential, activating regulatory element of the human *SIS/PDGFB* promoter in human megakaryocytes. Proc. Natl. Acad. Sci. USA 90: 7563-7567, 1993.
29. Jin H-M, Robinson DF, Liang Y, Fahl WE. *SIS/PDGF-B* promoter isolation and charcterization of regulatory elements necessary for basal expression of the *SIS/PDGF-B* gene in U2-OS osteosarcoma cells. J. Biol. Chem. 269: 28648-28654, 1994.
30. Khachigian LM, Fries JWU, Benz MW, Bonthron DT, Collins T. Novel *cis*-acting elements in the human platelet-derived growth factor B-chain core promoter that mediate gene expression in cultured vascular endothelial cells. J. Biol. Chem. 269: 22647-22656, 1994.
31. Dirks RPH, Jansen HJ, Van Gerven HJ, Onnekink C, Bloemers HPJ. *In vivo* footprinting and functional analysis of the human *c-sis/PDGF-B* gene promoter provides evidence for two binding sites for transcriptional activators. Nucl. Acids Res. 23: 1119-1126, 1995.
32. Dirks RPH, Jansen HJ, Gerritsma J, Onnekink C, Bloemers HPJ. Localization and functional analysis of DNaseI-hypersensitive sites in the human *c-sis/PDGF-B* gene transcription unit and its flanking regions. Eur. J. Biochem. 211: 509-519, 1993.
33. Franklin GC, Donovan M, Adam GIR, Holmgren L, Pfeifer-Ohlsson S, Ohlsson R. Expression of the human PDGF-B gene is regulated by both positively and negatively acting cell type-specific regulatory elements located in the first intron. EMBO J. 10: 1365-1375, 1991.

34. Dirks RPH, Jansen HJ, Onnekink C, de Jonge RJA, Bloemers HPJ. DNaseI-hypersensitive sites located far upstream of the human *c-sis/PDGF-B* gene comap with transcriptional enhancers and a silencer and are preceded by (part of) a new transcription unit. Eur. J. Biochem. 216: 487-495, 1993.
35. Wang C, Stiles CD. Platelet-derived growth factor α-receptor gene expression: isolation and characterization of the promoter and upstream regulatory elements. Proc. Natl. Acad. Sci. USA 91: 7061-7065, 1994.
36. Afink GB, Nistér M, Stassen BHGJ, Joosten PHLJ, Rademakers PJH, Bongcam-Rudloff E, Van Zoelen EJJ, Mosselman S. Molecular cloning and functional characterization of the human platelet-derived growth factor α-receptor gene promoter. Oncogene, 10: 1667-1672, 1995.
37. Mosselman S, Claesson-Welsh L, Kamphuis JS, Van Zoelen EJJ. Developmentally regulated expression of two novel platelet-derived growth factor α-receptor transcripts in human teratocarcinoma cells. Cancer Res. 54: 220-225, 1994.
38. Ballagi AE, Ishizaki A, Nehlin J-O, Funa K. Isolation and characterization of the mouse PDGF β-receptor promoter. Biochem. Biophys. Res. Commun. 210: 165-173, 1995.
40. Leof EB, Proper JA, Goustin AS, Shipley GD, DiCorleto PE, Moses HL. Induction of *c-sis* mRNA and activity similar to platelet-derived growth factor by transforming growth factor ß: a proposed model for indirect mitogenesis involving autocrine activity. Proc. Natl. Acad. Sci. USA 83: 2453-2457, 1986.
41. Janat MF, Liau G. Transforming growth factor-ß1 is a powerful modulator of platelet-derived growth factor action in smooth muscle cells. J. Cell. Physiol. 150: 232-242, 1992.
42. Haberstroh U, Zahner G, Disser M, Thaiss F, Wolf G, Stahl RAK. TGF-ß stimulates rat mesangial cell proliferation in culture: role of PDGF ß-receptor expression. Am. J. Physiol. 264: F199-F205, 1993.
43. Paulsson Y, Karlsson C, Heldin C-H, Westermark B. Density-dependent inhibitory effect of transforming growth factor-ß1 on human fibroblasts involves the down-regulation of platelet-derived growth factor α-receptors. J. Cell. Physiol. 157: 97-103, 1993.
44. Battegay EJ, Raines EW, Seifert RA, Bowen-Pope DF, Ross R. TGF-ß induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop. Cell 63: 515-524, 1990.
45. Drummond IA, Madden SL, Rohwer-Nutter P, Bell GI, Sukhatme VP, Rauscher FJ III. Repression of the insulin-like growth factor II gene by the Wilms' tumor suppressor WT 1. Science 257: 674-678, 1992.
46. Gashler AL, Bonthron DT, Madden SL, Rauscher FJ III, Collins T, Sukhatme VP. Human platelet-derived growth factor A chain is transcriptionally repressed by the Wilms tumor suppressor WT 1. Proc. Natl. Acad. Sci. USA 89: 10984-10988, 1992.
47. Wang ZY, Madden SL, Deuel TF, Rauscher FJ III. The Wilms' tumor gene product WT 1 represses transcription of the platelet-derived growth factor A-chain gene. J. Biol. Chem. 267: 21999-22002, 1992.
48. Werner H, Rauscher FJ III, Sukhatme VP, Drummond IA, Roberts CT Jr, LeRoith D. Transcriptional repression of the the insulin-like growth factor I receptor (IGF-I-R) gene by the tumor suppressor WT 1 involves binding to sequences both upstream and downstream of the IGF-I-R gene transcription start site. J. Biol. Chem. 269: 12577-12582, 1994.
49. Maheswaran S, Park S, Bernard A, Morris JF, Rauscher FJ III, Hill DE, Haber DA. Physical and functional interaction between WT 1 and p53 proteins. Proc. Natl. Acad. Sci. USA 90: 5100-5104, 1993.
50. Rodeck U, Bossler A, Kari C, Humphreys C, Györfi T, Maurer J, Thiel E, Menssen HD. Expression of the WT1 Wilms' tumor gene by normal and malignant human melanocytes. Int. J. Cancer 59: 78-82, 1994.
51. Pritchard-Jones K, Fleming S, Davidson D, Bickmore W, Porteous D, Gosden C, Bard J, Buckler A, Pelletier J, Housman D, Van Heyningen V, Hastie ND. The candidate Wilms' tumour gene is involved in genitourinary development. Nature 346: 194-197, 1990.
52. Pelletier J, Schalling M, Buckler AJ, Rogers A, Haber DA, Housman DE. Expression of the Wilms' tumour gene WT 1 in the murine urogenital system. Gene Dev. 5: 1345-1356, 1991.
53. Armstrong JF, Pritchard-Jones K, Bickmore WA, Hastie ND, Bard JBL. The expression of the Wilms' tumour gene, WT 1, in the developing mammalian embryo. Mech. Develop. 40: 85-97, 1992.
54. Park S, Schalling M, Bernard A, Maheswaran S, Shipley GC, Roberts D, Fletcher J, Shipman R, Rheinwald J, Demetri G, Griffin J, Minden M, Housman DE, Haber DA. The Wilms tumour gene WT 1 is expressed in murine mesoderm-derived tissues and mutated in a human mesothelioma. Nat. Genet. 4: 415-420, 1993.
55. Walker C, Rutten F, Yuan X, Pass H, Mew DM, Everitt J. Wilms' tumor suppressor gene expression in rat and human mesothelioma. Cancer Res. 54: 3101-3106, 1994.
56. Pelletier J, Bruening W, Kashtan CE, Mauer SM, Manivel JC, Striegel JE, Houghton DC, Junien C, Habib R, Fouser L, Fine RN, Silverman BL, Haber DA, Housman D. Germline mutations in the Wilms' tumour suppressor gene are associated with abnormal urogenital development in Denys-Drash syndrome. Cell 67: 437-447, 1991.
57. Bruening W, Bardeesy N, Silverman BL, Cohn RA, Machin GA, Aronson AJ, Housman D, Pelletier J. Germline intronic and exonic mutations in the Wilms' tumour gene (WT 1) affecting urogenital development. Nat. Genet. 1: 144-148, 1992.

58. Little MH, Williamson KA, Mannens M, Kelsey A, Gosden C, Hastie ND, Van Heyningen V. Evidence that WT 1 mutations in Denys-Drash syndrome patients may act in a dominant-negative fashion. Hum. Mol. Genet. 2, 259-264, 1993.
59. Quek HH, Chow VTK, Tock EPC. The third zinc finger of the WT 1 gene is mutated in Wilms' tumour but not in a broad range of other urogenital tumours. Anticancer Res. 13: 1575-1580, 1993.
60. Huff V, Jaffe N, Saunders GF, Strong LC, Villalba F, Ruteshouser E. WT1 exon 1 deletion/insertion mutations in Wilms tumor patients, associated with di- and trinucleotide repeats and deletion hotspot consensus sequences. Am. J. Hum. Genet. 56: 84-90, 1995.
61. Scucchi L, Mingazzini PL, DiStefano D, Falchi M, Camilli A, Vecchione A. Two cases of multicystic peritoneal mesothelioma: description and critical review of the literature. Anticancer Res. 14: 715-720, 1994.
62. Amin KM, Litzky LA, Smythe WR, Mooney AM, Morris JM, Mews DJY, Pass HI, Kari C, Rodeck U, Rauscher FJ III, Kaiser LR, Albelda SM. Wilms' tumor 1 susceptibility (WT1) gene products are selectively expressed in malignant mesothelioma. Am. J. Pathol. 146: 344-356, 1995.
63. Austin MB, Fechner RE, Roggli VL. Pleural malignant mesothelioma following Wilms' tumor. Am. J. Clin. Pathol. 86: 227-230, 1986.
64. Van der Meeren A, Seddon MB, Kispert J, Harris CC, Gerwin BI. Lack of expression of the retinoblastoma gene is not frequently involved in the genesis of human mesothelioma. Eur. Resp. Rev. 3: 177-179, 1993.
65. Shimizu E, Coxon A, Otterson GA, Steinberg SM, Kratzke RA, Kim YW, Fedorko J, Oie H, Johnson BE, Mulshine JL, Minna JD, Gazdar AF, Kaye FJ. RB protein status and clinical correlation from 171 cell lines representing lung cancer, extrapulmonary small cell carcinoma, and mesothelioma. Oncogene 9: 2441-2448, 1994.
66. Kafiri G, Thomas DM, Shepherd NA, Krausz T, Lane DP, Hall PA. p53 expression is common in malignant mesothelioma. Histopathology 21, 331-334, 1992.
67. Mayall FG, Goddard H, Gibbs AR. p53 immunostaining in the distinction between benign and malignant mesothelial proliferations using formalin-fixed paraffin sections. J. Pathol. 168, 377-381, 1992.
68. Ramael M, Lemmens G, Eerdekens C, Buysse C, Deblier I, Jacobs W, Van Marck E. Immunoreactivity for p53 protein in malignant mesothelioma and non-neoplastic mesothelium. J.Pathol. 168, 371-375, 1992.
69. Cagle PT, Brown RW, Lebovitz RM. p53 immunostaining in the differentiation of reactive processes from malignancy in pleural biopsy specimens. Hum. Pathol. 25: 443-448, 1994.
70. Battifora H. p53 immunohistochemistry: a word of caution. Hum. Pathol. 25: 435-4437, 1994.
71. Hall PA, Lane DP. p53 in tumour pathology: can we trust immunohistochemistry?-revisited! J. Pathol. 172: 1-4, 1994.
72. Cote RJ, Jhanwar SC, Novick S, Pellicer A. Genetic alterations of the p53 gene are a feature of malignant mesotheliomas. Cancer Res. 51: 5410-5416, 1991.
73. Metcalf RA, Welsh JA, Bennett WP, Seddon MB, Lehman TA, Pelin K, Linnainmaa K, Tammilehto L, Mattson K, Gerwin BI, Harris CC. p53 and Kirsten-ras mutations in human mesothelioma cell lines. Cancer Res. 52: 2610-2615, 1992.
74. Sekido Y, Pass HI, Bader S, Mew DJY, Christman MF, Gazdar AF, Minna JD. Neurofibromatosis type 2 (*NF2*) gene is somatically mutated in mesothelioma but not in lung cancer. Cancer Res. 55: 1227-1231, 1995.
75. Gibas Z, Li FP, Antman KH, Bernal S, Stahel RA, Sandberg AA. Chromosome changes in malignant mesothelioma. Cancer Genet. Cytogenet. 20: 191-201, 1986.
76. Stenman G, Olofsson K, Mansson T, Hagmar B, Mark J. Chromosomes and chromosomal evolution in human malignant mesotheliomas as reflected in sequential analysis of two cases. Hereditas 105: 233-239, 1986.
77. Popescu NC, Chahinian AP, DiPaolo JA. Nonrandom chromosome alterations in human malignant mesothelioma. Cancer Res. 48: 142-147, 1988.
78. Tiainen M, Tammilehto L, Mattson K, Knuutila S. Non-random chromosomal abnormalities in malignant pleural mesothelioma. Cancer Genet. Cytogenet. 33: 251-274, 1988.
79. Hagemeijer A, Versnel MA, Van Drunen E, Moret M, Bouts MJ, Van der Kwast ThH, Hoogsteden HC. Cytogenetic analysis of malignant mesothelioma. Cancer Genet. Cytogenet. 47: 1-28, 1990.
80. Meloni AM, Stephenson CF, Li FP, Sandberg AA. Del(6q) as a possible primary change in malignant mesothelioma. Cancer Genet. Cytogenet. 59: 57-61, 1992.
81. Center R, Lukeis R, Dietzsch E, Gillespie M, Garson OM. Molecular deletion of 9p sequences in non-small cell lung cancer and malignant mesothelioma. Genes Chrom. Cancer 7: 47-53, 1993.
82. Cheng JQ, Jhanwar SC, Lu YY, Testa JR. Homozygous deletions within 9p21-p22 identify a small critical region of chromosomal loss in human malignant mesotheliomas. Cancer Res. 53: 4761-4763, 1993.
83. Taguchi T, Jhanwar SC, Siegfried JM, Keller SM, Testa JR. Recurrent deletions of specific chromosomal sites in 1p, 3p, 6q, and 9p in human malignant mesothelioma. Cancer Res. 53: 4349-4355, 1993.

84. Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, Stockert E, Day RS III, Johnson BE, Skolnick MH. A cell-cycle regulator potentially involved in genesis of many tumor types. Science 264: 436-440, 1994.
85. Nobori T, Miura K, Wu J, Lois A, Takabayashi K, Carson D. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature 368: 753-756, 1994.
86. Hannon GJ, Beach D. $p15^{INK4B}$ is a potential effector of TGF-ß-induced cell cycle arrest. Nature 371: 257-261, 1994.
87. Cheng JQ, Jhanwar SC, Klein WM, Bell DW, Lee W-C, Altomare DA, Nobori T, Olopade OI, Buckler AJ, Testa JR. p16 alterations and deletion mapping of 9p21-p22 in malignant mesothelioma. Cancer Res. 54: 5547-5551, 1994.
88. Kim H-RC, Upadhyay S, Korsmeyer S, Deuel TF. Platelet-derived growth factor (PDGF) B and A homodimers transform murine fibroblasts depending on the genetic background of the cell. J. Biol. Chem. 269: 30604-30608, 1994.
89. Lee TC, Zhang Y, Aston C, Hintz R, Jagirdar J, Perle MA, Burt M, Rom WN. Normal human mesothelial cells and mesothelioma cell lines express insulin-like growth factor 1 and associated molecules. Cancer Res. 53: 2858-2864, 1993.
90. Craighead JE, Calore JD, Corson JM, Copeland KC, Maddox JC, Sporn MB. The pathogenetic role of growth factors in human and rat malignant mesothelioma. Eur. Resp. Rev. 3: 159-160, 1993.
91. Mórocz IA, Schmitter D, Lauber B, Stahel RA. Autocrine stimulation of a human lung mesothelioma cell line is mediated through the transforming growth factor α/epidermal growth factor receptor mitogenic pathway. Br. J. Cancer 70: 850-856, 1994.
92. Walker C, Everitt J, Ferriola PC, Stewart W, Mangum J, Bermudez E. Autocrine growth stimulation by transforming growth factor α in asbestos-transformed rat mesothelial cells. Cancer Res. 55: 530-536, 1995.
93. Surmacz E, Reiss K, Sell C, Baserga R. Cyclin D1 messenger RNA is inducible by platelet-derived growth factor in cultured fibroblasts. Cancer Res. 52: 4522-4525, 1992.
94. Winston JT, Pledger WJ. Growth factor regulation of cyclin D1 mRNA expression through protein synthesis-dependent and -independent mechanisms. Mol. Biol. Cell 4: 1133-1144, 1993.
95. Matsushime H, Roussel MF, Ashmun RA, Sherr CJ. Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell 65: 701-713, 1991.
96. Antoniades HN, Galanopoulos T, Nevill-Golden J, O'Hara CJ. Malignant epithelial cells in primary human lung carcinomas coexpress in vivo platelet-derived growth factor (PDGF) and PDGF receptor mRNA and their protein products. Proc. Natl. Acad. Sci. USA 89: 3942-3946, 1992.
97. Smythe WR, Kaiser LR, Hwang HC, Amin KM, Pilewski JM, Eck SJ, Wilson JM, Albelda SM. Succesful adenovirus-mediated gene transfer in an in vivo model of human malignant mesothelioma. Ann. Thorac. Surg. 57: 1395-1401, 1994.

SUMMARY

Human malignant mesothelioma is a mesodermally derived tumor of the coelomic cavities, that is most frequently found in the pleura. The incidence of malignant mesothelioma is strongly associated with asbestos exposure and, despite diminished asbestos use in the Netherlands, is still increasing. This is due to the long latency period (15-45 years) between asbestos exposure and the manifestation of the tumour. Although not much is known about the processes taking place during this period, it seems obvious that many growth-regulating molecules, that are known to contribute to uncontrolled growth of transformed cells, are involved. One of the growth factors implicated in the pathogenesis of malignant mesothelioma is platelet-derived growth factor (PDGF).

In this thesis, the expression of PDGF chains and receptors as well as their regulation were studied in normal and malignant mesothelial cells. The purpose of these studies was to further characterize the PDGF chain and receptor expression in normal and malignant mesothelial cells *in vitro* and *in vivo* and to get insight into the mechanisms resulting in the characteristic PDGF chain and receptor expression patterns in these cells.

A short summary on the differential expression patterns of PDGF chains and receptors in normal and malignant mesothelial cell lines is presented in **Chapter** 3.1. Mesothelioma cell lines demonstrated elevated PDGF A- and B-chain mRNA expression compared to normal mesothelial cell lines. Furthermore, cultured normal mesothelial cells were found to express PDGF α-receptor mRNA and only occasionally PDGF ß-receptor mRNA, whereas malignant mesothelioma cell lines produced PDGF ß-receptor mRNA and no PDGF α-receptor mRNA, as determined by Northern blot analysis. The corresponding proteins of these receptor mRNA molecules were also determined, using various assays. Immunocytochemical staining, immunoprecipitation, and radioreceptor analysis confirmed the presence of PDGF α-receptor proteins in normal mesothelial cells and PDGF ß-receptor proteins in mesothelioma cell lines (**Chapters** 3.1 and 3.2). In this way PDGF α-receptors could be detected in malignant mesothelioma cell lines as well using specific antibodies directed against this receptor subtype. Due to the fact that no antibodies specific for PDGF-AA or PDGF-BB are available for immunocytochemical staining, we could only conclude that normal as well as malignant mesothelial cell lines are capable of producing PDGF, the isoform being unidentified.

In **Chapter** 3.2 the *in vivo* pattern of PDGF and PDGF receptor protein expression was studied in malignant mesothelial cells derived from effusions and tumour tissues of malignant mesothelioma patients by immunostaining techniques. Both PDGF and PDGF ß-receptors were observed in malignant mesothelial cells derived from effusions, whereas PDGF α-receptor proteins could be identified in mesothelioma tissues as well. In non-malignant effusions, the mesothelial cells showed PDGF and PDGF α-receptor expression. The *in vivo* immunocytochemical pattern largely resembles the one obtained in *in vitro* growing normal and malignant mesothelial cells, indicating the relevance of the differential expression of PDGF chains and receptors in these cell lines. Furthermore, these data supports the hypothesis that mesothelioma cells are able to stimulate their

own growth in an autocrine way by producing both PDGF B-chain and its appropriate high-affinity PDGF ß-receptor.

In view of this putative autocrine loop in malignant mesothelial cells, regulation of PDGF chain and receptor production in normal and malignant mesothelial cell lines is an important topic to study. Regulation of PDGF A- and B-chain and PDGF α- and ß-receptor expression were thus analyzed in these cell lines. In **Chapter 4.1** we show by nuclear run off analysis that the increased PDGF A-chain expression in malignant mesothelial cells is most probably caused by an increased transcription rate, rather than an increased stability of the PDGF A-chain messenger. Consistent aberrations of chromosome region 7p22 (PDGF A-chain locus), or amplification or structural rearrangements of the PDGF A-chain locus do not seem to be responsible for this increased transcription rate. Both normal and malignant mesothelial cell lines were also analyzed for the presence of alternatively spliced PDGF A-chain transcripts containing exon 6 (giving rise to an extra 69 bases). This exon 6 sequence, encoding a cell retention signal, could hardly or not be demonstrated in the A-chain transcripts in either of the cell types.

The fact that in nuclear run off assays and on Northern blots of RNA from cycloheximide (CHX)-treated normal mesothelial cells no PDGF B-chain messengers could be identified, indicates that the undetectable PDGF B-chain mRNA expression in these cell lines is due to lack of transcription or, alternatively, to a low transcription initiation followed by rapid degradation (**Chapter 4.2**). As for the A-chain, chromosomal aberrations (22q) or gene rearrangements were not likely to be responsible for this effect. Activation of PDGF B-chain gene transcription was further studied using various assays. Basal B-chain promoter-induced CAT activity was observed in normal as well as malignant mesothelial cell lines, indicating that in principle normal mesothelial cells possess all necessary equipments for PDGF B-chain transcription initiation. Other (epigenetic) mechanisms may be involved in silencing of this transcription in the normal cells; CpG methylation and nucleosome positioning of the promoter region do not seem to be important in this respect. An *in vivo* footprint around bp -61, as observed in the promoter region of mesothelioma cells but not of normal mesothelial cells, demonstrates binding of a thus far unknown protein to promoter sequences in mesothelioma cells exclusively. Furthermore, mutation of the potential target binding sequence resulted in decreased reporter activity in the mesothelioma cells, indicating that the -64/-61 TCTC sequence presumably represents a region involved in transcription activation of PDGF B-chain expression. A DNaseI hypersensitive site around 9.9 kb upstream of the PDGF B-chain transcription start site, demonstrating enhancer activity in the mesothelioma cells, probably further enhances PDGF B-chain expression in these cells.

Analysis of regulation of PDGF α- and ß-receptor expression revealed that their messenger production is controlled at distinct levels (**Chapter 4.3**). The higher PDGF ß-receptor mRNA expression in mesothelioma cells, compared to their normal counterparts, is caused by a higher transcription initiation, as determined by nuclear run off analysis. PDGF α-receptor mRNA expression is controlled at the post-transcriptional level in these malignant cells, as they were found capable of synthesizing α-receptor transcripts, which could not be detected as steady-state messengers by Northern blot analysis. The fact that in RNA of CHX-treated mesothelioma cells no PDGF α-receptors were identified either, suggests that CHX-insensitive factors probably influence the stability of this messenger. Stimulation of normal and malignant mesothelial cells with

TGF-ß, which is one of the most extensively studied regulators of PDGF receptor expression, resulted in decreased PDGF α-receptor mRNA levels in normal mesothelial cell lines whereas increased PDGF ß-receptor levels were seen in 2 out of 6 mesothelioma cell lines. In these particular cell lines TGF-ß1 may thus enhance the autocrine PDGF loop via upregulation of PDGF ß-receptor mRNA levels.

In **Chapter** 5.1 the Wilms tumour (WT1) susceptibility gene product, which is suggested to be involved as a transcription factor in regulation of expression of PDGF A-chain mRNA, is introduced. Functional inactivation of the WT1 tumour suppressor gene is thought to contribute to formation of (part of) Wilms tumours. During development, this gene shows a limited expression, transcripts being found in kidney, spleen and the mesothelial linings of coelomic cavities. We observed WT1 mRNA expression in our normal mesothelial cell lines derived from adults (**Chapter** 5.2). Expression in the mesothelioma cell lines was highly variable and not related to the epithelial or fibrous morphology of the mesothelioma cell lines. Furthermore, an *in vitro* induced switch in morphology of the normal mesothelial cell lines did not lead to a change in WT1 level. In 4 out of 13 mesothelioma cell lines no WT1 transcripts were observed on Northern blots, suggesting that in these cases the tumour suppressor gene WT1 may contribute to the transformed character of the cell line. In the tested WT1 coding regions of WT1-expressing mesothelioma cell lines no point mutations (missense or nonsense) were found, whereas in all cases analyzed all four described WT1 splice variants could be identified.

In **Chapter** 5.2 the possible relationship between WT1 and PDGF A-chain mRNA levels in mesothelial cells is evaluated. The variation in WT1 mRNA levels in the mesothelioma cell lines did not correlate in any manner with the endogenous PDGF A-chain mRNA levels in these cell lines. In transfection studies, IGF2 has also been suggested to be regulated by WT1 proteins, but we could not find any correlation between WT1 levels and endogenous IGF2 mRNA levels in our cell lines. It may be that other factors influence expression of PDGF A-chain and IGF2 genes as well, thereby (partly) masking the transcription regulating properties of WT1.

In the studies described in this thesis we show that co-expression of PDGF B-chains and PDGF ß-receptors seems to be an important feature of malignant mesothelioma cells and that our mesothelial cell lines provide a good model system for studies on regulation of PDGF chain and receptor expression. From these studies we conclude that elevated expression levels of PDGF A-chain, B-chain and ß-receptor genes in the mesothelioma cell lines are primarily determined at the transcriptional level. Decreased PDGF α-receptor expression is probably due to messenger instability. No indications for regulation of PDGF A-chain transcription by WT1 have been found in mesothelial cell lines. The increased PDGF B-chain transcription in malignant mesothelioma cell lines is accompanied by binding of a factor to a TCTC sequence in the B-chain promoter region of these cell lines. A 5' located enhancer sequence may be involved in this increase as well. Future experiments should be directed towards identification and characterization of the factors involved in PDGF B-chain transcription via the beforementioned elements. Eventually this may result in new targets to interfere in the postulated autocrine PDGF loop, either experimentally or, possibly in future, therapeutically.

SAMENVATTING

Het humane maligne mesothelioom is een van het mesoderm afkomstige tumor van de lichaamsholten, die het meest frequent voorkomt in de pleura. De incidentie van het maligne mesothelioom is sterk geassocieerd met blootstelling aan asbest en neemt in ons land nog steeds toe, ondanks een verminderd gebruik van asbest. Dit wordt veroorzaakt door de lange latentietijd (15-45 jaren) tussen blootstelling aan asbest en het tot uiting komen van de tumor. Hoewel niet veel bekend is over de processen die tijdens deze periode plaatsvinden, lijken veel groeiregulerende moleculen, waarvan bekend is dat ze bijdragen aan ongeremde groei van getransformeerde cellen, hierbij betrokken zijn. Eén van de groeifactoren waarvan wordt verondersteld dat deze een rol speelt in de pathogenese van het maligne mesothelioom, is platelet-derived growth factor (PDGF).

In dit proefschrift werden de expressie van PDGF ketens en receptoren in normale en maligne mesotheelcellen, alsmede hun regulatie bestudeerd. Het doel van de studies was om de PDGF keten en receptor expressie in normale en maligne mesotheelcellen *in vitro* en *in vivo* verder te karakteriseren en om inzicht te krijgen in de mechanismen die resulteren in de karakteristieke PDGF keten en receptor expressiepatronen in die cellen.

In **Hoofdstuk** 3.1 zijn in een kort overzicht de onderling verschillende expressie patronen van PDGF ketens en receptoren in normale en maligne mesotheelcellen weergegeven. Mesothelioomcellijnen lieten verhoogde PDGF A- en B-keten expressie zien vergeleken met normaal-mesotheelcellijnen. Verder bleken gekweekte normale mesotheelcellen PDGF α-receptor mRNA en sporadisch PDGF ß-receptor mRNA tot expressie te brengen, terwijl maligne mesothelioomcellijnen PDGF ß-receptor mRNA en geen PDGF α-receptor mRNA produceerden, voor zover detecteerbaar met Northern blot assays. De eiwitten horende bij deze receptor mRNA moleculen werden eveneens bepaald. Immuuncytochemische kleuringen, immuunprecipitaties en radioreceptor assays bevestigden de aanwezigheid van PDGF α-receptor eiwitten in normale mesotheelcellen en van PDGF ß-receptor eiwitten in maligne mesothelioomcellijnen (**Hoofdstukken** 3.1 en 3.2). Gebruikmakend van specifieke antistoffen gericht tegen PDGF α-receptoren konden in maligne mesothelioomcellijnen eveneens PDGF α-receptoren gedetecteerd worden. Als gevolg van het feit dat er geen antistoffen, specifiek voor PDGF-AA of PDGF-BB, beschikbaar zijn, konden we alleen concluderen dat zowel normale als maligne mesotheelcellijnen in staat zijn PDGF te produceren.

In **Hoofdstuk** 3.2 werd het *in vivo* PDGF keten en expressiepatroon in maligne mesotheelcellen afkomstig van pleuravocht en tumorweefsel van mesothelioompatiënten bestudeerd met immuunkleuringen. Zowel PDGF als PDGF ß-receptoren werden gevonden in maligne mesotheelcellen afkomstig van pleuravocht, terwijl in mesothelioom tumorweefsel ook PDGF α-receptor eiwitten konden worden geïdentificeerd. In niet-maligne vochten brachten de mesotheelcellen PDGF en PDGF α-receptor tot expressie. Het *in vivo* immuuncytochemische patroon komt grotendeels overeen met dat verkregen in *in vitro* groeiende normale en maligne mesotheelcellen,

hetgeen de relevantie aangeeft van de onderling verschillende expressie van PDGF ketens en receptoren in deze cellijnen. Daarnaast ondersteunen deze gegevens de hypothese, dat mesothelioomcellen in staat zijn op een autocriene manier hun eigen groei te stimuleren door de produktie van PDGF B-keten en de bijpassende hoog affiene PDGF ß-receptor.

Met het oog op deze mogelijke autocriene groeistimulatie van maligne mesotheelcellen, is de regulatie van PDGF keten en receptor produktie in normale en maligne mesotheelcellen een belangrijk onderwerp van studie. De regulatie van PDGF A- en B-keten en PDGF α- en ß-receptor expressie werden daarom in deze cellijnen geanalyseerd. In **Hoofdstuk** 4.1 laten we met "nuclear run off" analyse zien, dat de verhoogde PDGF A-keten expressie in maligne mesotheelcellen hoogstwaarschijnlijk eerder door een verhoogde transcriptiesnelheid dan door verhoogde stabiliteit van het PDGF A-keten mRNA wordt veroorzaakt. Eenduidige afwijkingen van chromosoomregio 7p22 (PDGF A-keten locus) of amplificatie of structurele herschikkingen van het PDGF A-keten locus lijken niet verantwoordelijk te zijn voor deze verhoogde transcriptiesnelheid. De aanwezigheid van 'alternatively spliced' exon 6 (die zorgt voor 69 bp extra) bevattende PDGF A-keten transcripten werd eveneens geanalyseerd in zowel normale als maligne mesotheelcellen. Deze exon 6 sequentie, die codeert voor een 'cell retention' signaal, kon nauwelijks aangetoond worden in A-keten transcripten van één van deze beide celtypen.

Het feit dat in nuclear run off assays en op Northern blots met RNA van cycloheximide (CHX)-behandelde normale mesotheelcellen geen PDGF B-keten mRNA geïdentificeerd kon worden, geeft aan dat de niet-detecteerbare expressie van PDGF B-keten mRNA in deze cellijnen te wijten is aan afwezigheid van transcriptie of anders aan een geringe transcriptie-initiatie gevolgd door snelle afbraak (**Hoofdstuk** 4.2). Net als bij de A-keten, was het niet erg waarschijnlijk dat chromosomale afwijkingen (22q) of genherschikkingen voor dit effect verantwoordelijk waren. Activatie van PDGF B-keten transcriptie werd verder bestudeerd met diverse assays. Basale door de B-keten promoter geïnduceerde CAT activiteit werd zowel in normaal-mesotheelcellijnen als in maligne mesothelioomcellijnen gezien, wat aangeeft dat normale mesotheelcellen in principe de noodzakelijke uitrusting voor initiatie van PDGF B-keten transcriptie bevatten. Andere (epigenetische) mechanismen zouden betrokken kunnen zijn bij het stilleggen van deze transcriptie in de normale cellen; CpG-methylering and positionering van nucleosomen van het promotergebied lijken in dit verband niet belangrijk. Een *in vivo* footprint rond bp -61, zoals gevonden in de promoterregio van mesothelioomcellen, maar niet van normale mesotheelcellen, laat binding zien van een tot nu toe onbekend eiwit aan promotersequenties exclusief in mesothelioomcellen. Daarnaast resulteerde mutatie van de mogelijke target bindingssequentie in een verlaagde reporteractiviteit in de mesothelioomcellen, wat aangeeft dat de -64/-61 TCTC-sequentie wellicht een regio representeert die betrokken is bij transcriptie-activatie van PDGF B-keten expressie. Een DNaseI hypergevoelig gebied rond 9.9 kb upstream van de PDGF B-keten transcriptie-startplaats dat enhanceractiviteit vertoonde in mesothelioomcellen, verhoogt waarschijnlijk verder PDGF B-keten expressie in deze cellen.

Analyse van de regulatie van PDGF α- en ß-receptor expressie maakte duidelijk dat hun messengerproduktie op verschillende niveaus wordt gecontroleerd (**Hoofdstuk** 4.3).

De hogere PDGF ß-receptor mRNA expressie in maligne mesothelioomcellen, vergeleken met hun normale tegenhangers, wordt veroorzaakt door een hogere transcriptie initiatie, zoals bepaald met "nuclear run off" analyse. PDGF α-receptor mRNA expressie wordt gecontroleerd op een post-transcriptioneel niveau in deze maligne cellen, aangezien ze in staat werden gevonden tot synthese van α-receptor transcripten die niet als steady-state mRNA via Northern blot analyse kon worden gedetecteerd. Het feit dat in RNA van CHX-behandelde maligne mesothelioomcellen eveneens geen PDGF α-receptors werden geïdentificeerd, suggereert dat CHX-ongevoelige factoren waarschijnlijk de stabiliteit van dit mRNA beïnvloeden. Stimulering van normale en maligne mesotheelcellen met TGF-ß1, dat een van de meest uitgebreid bestudeerde regulatoren van PDGF receptorexpressie is, resulteerde in verlaagde PDGF α-receptor mRNA niveaus in de normale mesotheelcellijnen, terwijl verhoogde PDGF ß-receptorniveaus werden gezien in 2 van de 6 mesothelioomcellijnen. In die betreffende cellijnen zou TGF-ß1 dus de autocriene PDGF groeistimulatie via verhoging van PDGF ß-receptor mRNA niveaus kunnen versterken.

In **Hoofdstuk** 5.1 wordt het Wilms tumor (WT1) "susceptibility" gen produkt, waarvan gesuggereerd wordt dat het als transcriptiefactor betrokken is bij regulatie van PDGF A-keten mRNA expressie, geïntroduceerd. Functionele inactivatie van het WT1 tumor suppressorgen wordt verondersteld bij te dragen aan de vorming van (een gedeelte van) Wilms tumoren. Gedurende de ontwikkeling vertoont dit gen een beperkte expressie waarbij transcripten worden gevonden in de nier, milt en mesotheliale bekleding van de coeloomholten. Wij vonden WT1 mRNA expressie in onze van volwassenen afkomstige normaal-mesotheelcellijnen (**Hoofdstuk** 5.2). Expressie in de mesothelioomcellijnen was sterk variabel en niet gerelateerd aan de epitheliale of fibreuze morfologie van deze cellijnen. Daarnaast leidde een *in vitro* geïnduceerde verandering in de morfologie van de normaal-mesotheelcellijnen niet tot een verandering in WT1 niveau. In 4 van de 13 mesothelioomcellijnen werd geen WT1 mRNA gevonden op Northern blots, hetgeen suggereert dat het tumor suppressorgen WT1 bij zou kunnen dragen aan het getransformeerde karakter van de cellijn in die gevallen. In de onderzochte WT1 coderende gebieden van WT1 tot expressie brengende mesothelioomcellijnen werden geen puntmutaties ("missense" of "nonsense") gevonden, terwijl alle vier beschreven WT1 splice varianten in alle gevallen geïdentificeerd konden worden.

In **Hoofdstuk** 5.2 wordt de mogelijke relatie tussen WT1 en PDGF A-keten mRNA niveaus in mesotheelcellen geëvalueerd. De variatie in WT1 mRNA niveaus in de mesothelioomcellijnen correleerde op geen enkele manier met de endogene PDGF A-keten mRNA niveaus in deze cellijnen. In transfectiestudies is gesuggereerd dat IGF2 eveneens door WT1 eiwitten wordt gereguleerd, maar wij konden geen correlatie vinden tussen WT1 niveaus en endogene IGF2 niveaus in onze cellijnen. Het zou zo kunnen zijn dat andere factoren de expressie van PDGF A-keten en IGF2 genen eveneens beïnvloeden, daarmee (gedeeltelijk) de transcriptieregulerende eigenschappen van WT1 verhullend.

De in dit proefschrift beschreven studies tonen aan dat co-expressie van PDGF B-ketens en PDGF ß-receptoren een belangrijk kenmerk van maligne mesothelioomcellen is en dat onze mesotheelcellijnen een goed modelsysteem vormen voor studies naar

regulatie van PDGF keten en receptor expressie. Op basis van deze studies concluderen we dat de verhoogde expressieniveaus van PDGF A-keten, B-keten en ß-receptor genen in de mesothelioomcellijnen primair bepaald worden op transcriptieniveau. Verlaagde PDGF α-receptor expressie is waarschijnlijk te wijten aan instabiliteit van het betreffende mRNA Er zijn geen aanwijzingen gevonden voor regulatie van PDGF A-keten transcriptie door WT1 in mesotheel cellijnen. De verhoogde B-keten transcriptie in maligne mesothelioomcellijnen gaat gepaard met binding van een factor aan een TCTC-sequentie in de B-keten promoterregio van die cellijnen. Een 5' gelegen enhancersequentie zou eveneens bij deze toename betrokken kunnen zijn. Toekomstige experimenten zullen gericht moeten zijn op het identificeren en karakteriseren van de factoren die via de genoemde elementen bij de PDGF B-keten transcriptie betrokken zijn. Uiteindelijk zou dit kunnen leiden tot nieuwe aangrijpingspunten om experimenteel of, wellicht in de toekomst, therapeutisch in te grijpen in de gepostuleerde autocriene PDGF groeistimulatie.

SAMENVATTING VOOR DE GEÏNTERESSEERDE LEEK

Een introductie in het maligne mesothelioom, groeifactoren en transcriptie

Kanker is een ziektebeeld dat gekenmerkt wordt door een ongeremde groei van cellen op een specifieke plaats in het lichaam. Kanker kan ontstaan in diverse organen (bijv. dikke darm, borst, huid en longen) of in het bloed of beenmerg. Het maligne mesothelioom is een kwaadaardige (maligne) vorm van kanker van de vliezen die de twee grote lichaamsholten (de borst- en buikholte) bekleden. Uit diverse studies is bekend, dat het voorkomen van een maligne mesothelioom sterk geassocieerd is met blootstelling aan asbestvezels. Asbest is een natuurlijk voorkomend materiaal, dat vanwege zijn bijzondere eigenschappen (o.a. brandwerend en isolerend) in het verleden zeer veel toegepast is in scheepsbouw, isolatie van gebouwen en remleidingen van auto's. Ondanks het feit dat het gebruik van asbest sinds 1993 is verboden, is er nog steeds een toename te zien in het aantal mensen dat een maligne mesothelioom krijgt. Dit heeft te maken met de lange tijd tussen blootstelling aan asbest en de uiteindelijke eerste ziekteverschijnselen en diagnose. Deze latentietijd kan variëren van 15 tot 45 jaar. De toename in het aantal mesothelioompatiënten zal naar verwachting doorgaan tot 2005-2010.

Hoewel over de precieze processen die een rol spelen bij het ontstaan en de verdere ontwikkeling van een maligne mesothelioom nog niet veel bekend is, wordt uit diverse onderzoeken duidelijk dat zgn. groeifactoren een belangrijke rol spelen. Groeifactoren zijn kleine eiwitten die door diverse celtypen geproduceerd en (meestal) uitgescheiden kunnen worden. Cellen die in het bezit zijn van zgn. receptoren zullen vervolgens die groeifactoren kunnen binden. Uiteindelijk leidt zo'n binding voor de betreffende cel tot een signaal om zich te gaan delen.

De produktie van een eiwit in het algemeen, en daarmee dus ook van groeiregulerende eiwitten of hun receptoren, vindt plaats in een aantal stappen. In het DNA ligt de blauwdruk voor de vorm en structuur van het eiwit opgeslagen. Via een complex proces dat transcriptie wordt genoemd, wordt deze gecodeerde informatie overgeschreven naar een tussenvorm, het mRNA. Dit proces is een noodzakelijke stap die voorafgaat aan de uiteindelijke vertaling van de (gekopieerde) mRNA-code naar eiwit. Dat laatste proces van vertaling heet translatie. Hoewel in diverse processen de eiwitten de uiteindelijke werkzame stoffen zijn, is met name het wel of niet overschrijven van de code van DNA naar mRNA in eerste instantie bepalend voor de produktie van eiwitten. Bestudering van de manier waarop het proces van transcriptie van een bepaald stuk DNA wordt geregeld is dan ook onderwerp van veel studies.

PDGF en het maligne mesothelioom

De studies, zoals die staan beschreven in dit proefschrift, gaan over de regulatie van transcriptie van een bepaalde groeifactor, PDGF ("platelet-derived growth factor"; ofwel de groeifactor zoals die het eerst uit de bloedplaatjes werd gezuiverd), in maligne mesothelioomcellen. Voor deze studies zijn maligne mesothelioomcellijnen gebruikt; dat

zijn van mesothelioompatiënten afkomstige kankercellen die in kweekflesjes groeien. Daarnaast is voor een goede vergelijking gebruik gemaakt van gekweekte normaal mesotheelcellen als gezonde tegenhangers van mesothelioomcellen. Uit het bovenstaande is duidelijk wat een groeifactor is en wat een maligne mesothelioom is. De gedachte (hypothese) achter ons onderzoek was dat de door maligne mesothelioomcellen geproduceerde groeifactor (PDGF) door diezelfde cellen wordt herkend en gebonden via hun receptoren en dat dit leidt tot een signaal om te gaan delen. Samengevat: mesothelioomcellen worden dus in staat geacht hun eigen celdeling te stimuleren. In jargon heet dat: autocriene stimulatie van groei.

Uit diverse studies komen aanwijzingen die een dergelijk model ondersteunen; ook ons onderzoek ondersteunt dit model. Van de groeifactor PDGF bestaan twee soorten ketens, de zgn. A- en B-ketens, terwijl er ook twee PDGF receptorsoorten bekend zijn, de α- en ß-receptoren. Uit onderzoek is bekend dat met name de combinatie PDGF B-keten/PDGF ß-receptor leidt tot heel sterke binding en dus tot delingssignalen voor de cel. Inventarisatie van de mesothelioomcellijnen laat zien, dat deze zowel PDGF A- als B-keten mRNA maken, evenals PDGF ß-receptor mRNA. Hun gezonde tegenhangers, de normale mesotheelcellen, produceren alleen PDGF A-keten en PDGF α-receptor mRNA (**Hoofdstuk** 3.1). De voor maligne mesothelioomcellen karakteristieke produktie van PDGF B-keten en PDGF ß-receptor mRNA wordt daarnaast ook teruggevonden in de produktie van de betreffende eiwitten. Zowel gekweekte mesothelioomcellijnen als niet-gekweekte cellen van mesothelioompatiënten blijken PDGF en PDGF ß-receptoren te produceren (**Hoofdstuk** 3.2).

PDGF en het maligne mesothelioom: transcriptie

Uitgaande van de hiervoor beschreven karakteristieke produktie van PDGF ketens en receptoren door maligne mesothelioomcellen en gezonde mesotheelcellen zijn vervolgens studies naar de regulatie van deze karakteristieke produktie gestart. De verhoogde produktie van PDGF A-keten mRNA in maligne mesothelioomcellen blijkt het gevolg te zijn van een hogere snelheid van transcriptie (**Hoofdstuk** 4.1). De hoge PDGF B-keten mRNA produktie wordt veroorzaakt doordat mesothelioomcellen voor dit gedeelte DNA eveneens een hoge transcriptiesnelheid vertonen. Dat het onmogelijk bleek in normale mesotheelcellen PDGF B-keten mRNA aan te tonen, lijkt het gevolg te zijn van het ontbreken van PDGF B-keten transcriptie in deze cellen (**Hoofdstuk** 4.2). Bepaalde (nog niet nader geïdentificeerde) eiwitten, die binden aan gebieden in het DNA met een regulerende functie, lijken betrokken te zijn bij dit verschil in PDGF B-keten transcriptie tussen normale mesotheelcellen en maligne mesothelioomcellen. Duidelijk is geworden dat een van deze regulerende DNA gebieden zich dicht in de buurt van het PDGF B-keten DNA (d.w.z. in het zgn. promotergebied) bevindt en de ander in een wat verder gelegen gebied (**Hoofdstuk** 4.2). Bij de PDGF receptoren bleek iets anders aan de hand. Ondanks het feit dat in mesothelioomcellen geen PDGF α-receptor mRNA werd gevonden (zie eerder), blijkt dat deze cellen, net zoals mesotheelcellen, wel degelijk PDGF α-receptor mRNA kunnen maken. Het uiteindelijke verschil tussen de normale en de kankercellen wordt veroorzaakt doordat dit PDGF α-receptor mRNA in mesothelioomcellen snel afgebroken wordt, maar in normale mesotheelcellen niet. Normale mesotheelcellen blijken verder niet in staat tot PDGF ß-receptor transcriptie,

terwijl mesothelioomcellen wel transcriptie van dit DNA gedeelte vertonen (**Hoofdstuk 4.3**).

Tenslotte is van een bepaald eiwit, het WT1 (genoemd naar Wilms tumoren, tumoren van de nieren, waar dit eiwit het eerst is beschreven) tumorsuppressor eiwit, gesuggereerd dat het een rol zou spelen als remmend eiwit bij PDGF A-keten transcriptie. Het kenmerk van een tumor suppressoreiwit is, dat het in normale, gezonde cellen voor onderdrukking (suppressie) van de tumorvorming (kankervorming) zorgt en dat het wegvallen van die remmende functie in bepaalde situaties de betreffende cel tot een kankercel kan maken. Hoewel deze bevindingen erg goed zouden kunnen passen in het proces van tumorvorming in maligne mesothelioomcellen, blijkt experimenteel dat WT1 mRNA slechts in een kleine minderheid van mesothelioomcellen afwezig is, terwijl de rol van WT1 als regulator van PDGF A-keten transcriptie ook niet heel duidelijk is in mesothelioomcellen.

Concluderende opmerkingen

In dit proefschrift wordt een aantal studies beschreven, die verdere aanwijzingen hebben opgeleverd voor de belangrijke rol die de groeifactor PDGF speelt bij het maligne mesothelioom. Uit die studies komt de suggestie dat met name de gecombineerde produktie van PDGF B-ketens en PDGF ß-receptoren door mesothelioomcellen belangrijk is. Daarnaast wordt beschreven hoe de karakteristieke PDGF keten produktie en PDGF receptor produktie van mesotheelcellen en maligne mesothelioomcellen worden geregeld. In geval van de PDGF B-keten produktie bij mesothelioomcellen is een aantal gebieden rond het PDGF B-keten DNA gedeelte geïdentificeerd, waar eiwitten binden, die een essentiële rol lijken te spelen in PDGF B-keten transcriptie.

Verdere studies zullen nu moeten uitwijzen in hoeverre ingrijpen in de produktie en/of werking van de groeifactor PDGF (m.n. PDGF B-keten of PDGF ß-receptor) leidt tot een remming van groei van de kankercellen. Wellicht vormen de beschreven regulerende gebieden een goed aangrijpingspunt voor een dergelijke strategie.

ABBREVIATIONS

a.a.	:	amino acid
Act D	:	actinomycin D
bp	:	basepair
BSA	:	bovine serum albumin
BWS	:	Beckwith-Wiedemann syndrome
cDNA	:	complementary DNA
CAT	:	chloramphenicol acetyl transferase
CHX	:	cycloheximide
c-*onc*	:	cellular oncogene
CSF	:	colony stimulating factor
DDS	:	Denys-Drash syndrome
DH	:	DNaseI hypersensitive
DNA	:	deoxyribonucleic acid
EGF	:	epidermal growth factor
ECM	:	extracellular matrix
EMA	:	epithelial membrane antigen
FAK	:	focal adhesion kinase
FCS	:	fetal calf serum
FITC	:	fluorescein isothiocyanate
GAP	:	GTPase activating protein
GAPDH	:	glyceraldehyde-3-phosphate dehydrogenase
Grb	:	growth factor receptor bound
GTP	:	guanosine triphosphate
HC	:	hydrocortisone
IF	:	immunofluorescence
IGF	:	insulin-like growth factor
kb	:	kilobase
kDa	:	kilo Dalton
LOH	:	loss of heterozygosity
M	:	mesothelial cells
Me	:	malignant mesothelioma cells
Mero	:	malignant mesothelioma cell line
mRNA	:	messenger RNA
NA	:	not applicable
ND	:	not determined
NM	:	normal mesothelial cell line
p	:	short arm of chromosome
P13-K	:	phosphatidyl inositol 3 kinase
PBS	:	phosphate-bufferred saline
PCR	:	polymerase chain reaction
PDGF	:	platelet-derived growth factor
PLCγ	:	phospholipase cγ
PTP	:	protein tyrosine phosphatase
q	:	long arm of chromosome
RNA	:	ribonucleic acid
RT	:	reverse transcription or room temperature
SH2	:	Src homology 2
SSC	:	standard saline citrate
TGF	:	transforming growth factor
TPA	:	12-O-tetradecanoylphorbol 13-acetate
TRITC	:	tetramethylrhodamine isothiocyanate
UTS	:	untranslated sequence
v-*onc*	:	viral oncogene
WAGR (syndrome)	:	Wilms tumour, aniridia, genitourinary abnormalities, mental retardation-syndrome
WT	:	Wilms tumour

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Ton

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sinds april 1995	:	ontwikkeling en toepassing van moleculair-immunologische diagnostiek binnen de afdeling Immunologie van het Academisch Ziekenhuis Rotterdam - Dijkzigt

LIST OF PUBLICATIONS

1. Versnel MA, Bouts MJ, Langerak AW, van der Kwast ThH, Hoogsteden HC, Hagemeijer A, Heldin C-H. Hydrocortisone-induced increase of PDGF ß-receptor expression in a human malignant mesothelioma cell line. Exp. Cell Res. 200: 83-88, 1992.

2. Langerak AW, Dirks RPH, Versnel MA. Splicing of the platelet-derived growth factor A-chain mRNA in human malignant mesothelioma cell lines and regulation of its expression. Eur. J. Biochem. 208: 589-596, 1992.

3. Langerak AW, Vietsch H, Bouts MJ, Hagemeijer A, Versnel MA. A spontaneously *in vitro* transformed mesothelial cell line has a similar pattern of PDGF chain and PDGF receptor expression to malignant mesothelioma cell lines. Eur. Respir. Rev. 3: 170-174, 1993.

4. Versnel MA, Langerak AW, van der Kwast ThH, Hoogsteden HC, Hagemeijer A. Expression of PDGF chains and PDGF receptors in human malignant and normal mesothelial cell lines. Eur. Respir. Rev. 3: 186-188, 1993.

5. Versnel MA, Haarbrink M, Langerak AW, de Laat PAJM, Hagemeijer A, van den Berg L, Schrier PI. Human ovarian tumors from epithelial origin express PDGF *in vitro* and *in vivo*. Cancer Genet. Cytogenet. 73: 60-64, 1994.

6. Langerak AW, Williamson KA, Miyagawa K, Hagemeijer A, Versnel MA, Hastie ND. Expression of the Wilms' tumor gene *WT1* in human malignant mesothelioma cell lines and relationship to platelet-derived growth factor A and insulin-like growth factor 2 expression. Genes Chromosom. Cancer 12: 87-96, 1995.

7. Langerak AW, de Laat PAJM, van der Linden-van Beurden CAJ, Delahaye M, van der Kwast ThH, Hoogsteden HC, Benner R, Versnel MA. Expression of PDGF and PDGF receptors in mesothelioma *in vitro* and *in vivo*. J. Pathol., in press.

8. Langerak AW, van der Linden-van Beurden CAJ, Versnel MA. Regulation of differential expression of PDGF and ß-receptor mRNA in normal and malignant human mesothelial cell lines. Biochim. Biophys. Acta, in press.

9. Langerak AW, Dirks RPH, de Laat PAJM, Prins J-B, Bloemers HPJ, Versnel MA. Mechanism of transcriptional activation of PDGF B-chain expression in human malignant mesothelioma cell lines. Submitted for publication.

10. Hoogsteden HC, Langerak AW, van der Kwast ThH, Versnel MA, van Gelder T. Malignant pleural mesothelioma. Submitted for publication.

11. Van Dongen JJM, van den Beemd MWM, Schellekens M, Wolvers-Tettero ILM, Langerak AW, Groeneveld K. Analysis of malignant T-cells with the Vβ antibody panel. Immunologist, in press.