The IGF system during growth and differentiation of the mouse

Het IGF systeem tijdens de groei en differentiatie van de muis

Proefschrift

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Wat achter ons ligt en wat voor ons ligt,

is niets vergeleken met wat er in ons ligt

Ralph Waldo Emerson

Contents

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List of abbreviations

AAS	aminopropyl trioxysilane
ABC	avidine-biotine complex
AER	apical ectodermal ridge
ALS	acid labile subunit
BMP	bone morphogenetic protein
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CRF	chronic renal failure
DIG	digoxigenin
DNA	deoxyribonucleic acid
dpc	days post coitum
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
FGF	fibroblast growth factor
FSH	follicle stimulating hormone
Gdf-5	growth and differentiation factor-5
GH	growth hormone
hCG	human chorionic gonadotropin
HOX	homeobox
IDDM	insulin dependent diabetes mellitus
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IGFR	insulin like growth factor receptor
ISH	*in situ* hybridization
kDa	kilo Dalton
KLH	keyhole limpet hemocyanin
MEL	murine erythroleukaemia
mRNA	messenger ribonucleic acid
M6PR	mannose-6-phosphate receptor
NBT	4-nitroblue tetrazolium chloride
NSILA	nonsuppressible insulin-like activity
PAGE	polyacrylamide-gel electrophoresis
PAP	peroxidase-anti-peroxidase
PBS	phosphate buffered saline

List of abbreviations (continued)

PTH	parathyroid hormone
PVA	polyvinylalcohol
PZ	progress zone
RA	retinoic acid
RGD	Arg-Gly-Asp
RIA	radioimmunoassay
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
SEM	standard error of means
SHH	sonic hedgehog
SSC	standard saline citrate
TdT	terminal deoxynucleotidyl transferase
TGFβ	transforming growth factor beta
Tunel	TdT-mediated dUTP nick end labeling

Chapter 1

General Introduction

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General introduction

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The insulin-like growth factors (IGFs) were first discovered in 1957 by Salmon and Daughaday (1957). They found that *in vivo* administration of growth hormone (GH) induced a serum factor capable of stimulating sulfate uptake in cartilage. In 1972 this 'sulfation factor' was renamed somatomedin (mediator of the effects of somatotropin, GH) and classified as a growth factor (Daughaday et al., 1972). At the same time, a compound named NSILA (nonsuppressible insulin-like activity) was found (Froesch et al., 1966). NSILA and somatomedin each stimulated glucose incorporation into fat and sulfate incorporation into cartilage (Froesch et al., 1976). The amino acid sequence of NSILA showed 48% homology with human pro-insulin. Therefore it was called insulin-like growth factor-I (IGF-I) (Rinderknecht and Humbel, 1978a). A second bioactive insulin-like molecule appeared to be similar, but not identical, and was named IGF-II (Rinderknecht and Humbel, 1978b). Somatomedin appeared to be identical to IGF-I (Svoboda et al., 1980).

Now, over forty years later, *in vivo* studies, gene expression experiments, and determination of signalling pathways have provided more insight into IGF actions. IGF receptors and IGF binding proteins (IGFBPs) have been characterized (Chernausek et al., 1981; Kasuga et al., 1981; Brinkman et al., 1988; Binkert et al., 1989; Margot et al., 1989; Shimasaki, 1989; Murphy et al., 1990; Shimasaki, 1990; 1991a/b; Schuller et al., 1994). This, and the recent discovery of IGFBP related proteins, have shown the complexity of the IGF system (Oh et al., 1996). It is clear that the IGFs, together with the other IGF system compounds, play a pivotal role in body and organ development and growth.

Therefore, this chapter will review in more detail characteristics of the separate components of the IGF system. Then, focus is put on the IGF system during mouse development. A summary of the data generated to elucidate the functions of the IGF system during mouse development is described. Refined techniques enabling detailed localization and (semi)quantification of expression and specific gene manipulation studies have shed new light on the spatial and temporal interrelationships of the actions of the members of the IGF system, their role during preimplantation, embryogenesis, and fetal development. It is important to bear in mind that the IGF system represents only one group from a large list of growth factors and cytokines expressed in the female reproductive tract and embryo/fetus, and that these factors together determine the complex processes taking place during development. Beside the growth related events taking place during embryogenesis, also programmed cell death or apoptosis, is a prominent aspect. Therefore, the possible function of the IGF system in the regulation of apoptosis will also be discussed.

IGF-I

The insulin-like growth factors are low molecular weight single chain polypeptides with structural homology to each other and to proinsulin. Both IGFs are synthesized as precursors, containing a signal peptide, the mature peptide and a trailer peptide. The IGF-I polypeptide is 70 amino acid residues long. IGF-I is a single gene product (approximately 95 kb long), and the gene contains six exons. Exons 5 and 6 are used alternately, leading to the two different precursors IGF-Ia and IGF-Ib. Transcription of the IGF-I gene leads to

multiple mRNAs with varying sizes, which are expressed in a variety of cell types where they serve as ubiquitous cellular growth promoters.

The major regulators of IGF-I expression are nutritional status and GH (Rotwein, 1991). Furthermore, IGF-I is regulated by many other factors, in a tissue specific manner. Examples are the estrogen regulation of IGF in the uterus, or the effect of parathyroid hormone (PTH) in cartilage.

During development many changes in IGF expression occur, which are mediated by a variety of unknown factors. This implicates that the somatomedin hypothesis remains partly valid. This hypothesis predicted control of IGF-I levels by GH and direct IGF-I action at the cellular level (Daughaday et al., 1972). Originally, somatomedins (IGFs) were thought to function primarily as circulating hormones secreted by the liver in response to GH. Indeed, IGF-I can be seen as a hormone; blood levels often reflect alterations in endocrine physiology (e.g., low in hypopituitarism, high in acromegaly).

Furthermore, most IGF-I is found complexed to binding proteins (IGFBPs), which prolong the half life of IGF-I and distribute IGF into many tissues. Liver is the main IGF-I producing organ and also expresses most of the IGFBPs. mRNA expression of IGF-I in liver is regulated by growth hormone (GH) and nutrition, which is also true for some of the IGFBPs (Kelley et al., 1996).

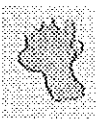
During embryonic and fetal development, the IGFs are seen as local growth factors. The growth promoting effects of IGF-I are also evident postnatally. The endocrine function of IGF-I seems to be more important with progressing maturation (D'Ercole, 1996). The fact that both IGFs are expressed in a wide variety of tissues and cell types, and their direct secretion without primary storage in secretory granules show their main function as circulating growth factors. After the discovery of the IGFs, their biological significance was soon expanded to include stimulation of DNA synthesis, proteoglycan synthesis, glycosaminoglycan synthesis and protein synthesis. IGF stimulatory actions on cell proliferation are best characterized, while more data on the complementary, inhibiting effects on programmed cell death now become apparent (see below).

IGF-II

Similar to IGF-I, the 67 amino acid IGF-II polypeptide is a single gene product. The gene spans 35 kb and contains multiple exons. Transcription results in mRNAs of multiple sizes, due to alternate exon usage. In the mouse, the IGF-II gene is paternally imprinted, resulting in preferential expression of the paternal IGF-II gene during embryonic/fetal development. Hence, offspring with the disrupted gene from the father is growth retarded (Barlow et al., 1991). The mechanism of imprinting is not completely understood and appears to be tissue specific, as no imprinting of IGF-II occurs in the brain (Polychronakos, 1994).

Like IGF-I, IGF-II is widely expressed throughout organs and tissues of the body, and its contribution to developmental growth is highest during the second half of gestation (D'Ercole, 1996).

The knowledge of regulation of IGF transcription is only rudimentary. IGF genes do not contain classical 5' promoters, enhancers and regulatory elements (D'Ercole 1996). Many undefined factors influence IGF expression during embryonic and fetal development. IGF-II transcripts are more abundant than IGF-I transcripts, in general, in embryonic and fetal tissues. Postnatally, IGF-II levels decrease dramatically (D'Ercole, 1996). The changing sites and ontogeny of IGF expression during development points to a tissue specific regulation.



In addition, the understanding of the role of IGF-II in growth promoting actions is still incomplete.

Besides its growth enhancing properties, IGF-II does not seem to have endocrine properties. Its high blood levels are not highly regulated, and in contrast to IGF-I this factor seems to work merely locally, as was concluded from experiments with mice overexpressing IGF-II in certain tissues (Wolf et al., 1994). Studies on mice with ablated IGF-I, IGF-II and/or IGF receptor genes, indicate that IGF-II actions are mediated mostly by the type I IGF receptor (DeChiara et al., 1990; Liu et al., 1993b). More details will be provided elsewhere in this chapter.

IGF receptors

The IGFs mediate their effects by binding to two specific IGF receptors on target cell surfaces. The transmembrane type I receptor has an extracellular α-subunit with binding capacity and a β-subunit with tyrosine kinase activity. The α- and β-subunit are linked by disulfide bonds to form an αβ-half-receptor. In turn, two of these αβ-half-receptors are linked by disulfide bonds between the two α-units, to form the mature $\alpha_2\beta_2$-holoreceptor (Chernausek et al., 1981).

Tyrosine kinase activity resides in the cytoplasmatic β-domain. Similar to the homology between insulin and the IGFs, the type I IGF receptor shows resemblance with the insulin receptor, which is also composed of 4 subunits and contains tyrosine kinase activity. Binding affinity for the type I receptor is greatest for IGF-I, least for insulin and intermediate for IGF-II (D'Ercole, 1996).

Binding of IGFs to the extracellular α-subunit induces conformational changes in the receptor, resulting in autophosphorylation of the β-subunit. Several distinct pathways are involved in IGF signalling, which is likely to be cell specific.

The type II receptor is a very different integral membrane molecule; it is identical to the cation-independent mannose-6-phosphate receptor, which is a single transmembrane protein that does not have tyrosine kinase activity. When localized on the membranes of the trans-Golgi network, it functions by translocating newly synthesized lysosomal enzymes into endosomes by endocytosis (Morgan et al.,1987). On the cell surface it binds mannose-6-phosphate containing extracellular glycoproteins (e.g. proliferin, thyroglobulin and the latent form of transforming growth factor-β). The type II IGF/Man-6-P receptor binds IGF-II with high affinity, while the affinity for IGF-I is two magnitudes of order lower and binding to insulin does not occur (Tong et al., 1988; 1989). The binding sites for IGF-II and Man-6-P or Man-6-P-containing glycoproteins differ and binding of these two different types of proteins can occur simultaneously. Rather than having a role in signal transduction it appears more likely that the type II IGF receptor functions as a clearance receptor for IGF-II (Oka et al., 1985; Czech et al., 1989, Filson et al., 1993). It is less clear whether this receptor is mediating any of the biological effects of IGF-II.

Table 1.
Characteristics of the six mIGFBPs

mIGFBP	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6
mRNA (kb)	1.5	2.0	2.6	2.6	5.0	1.1
protein (kDa)	~30	~31	~43/~45*	~24/~28**	~29	~30
cystein residues	18	18	18	20	18	16
binding preference	IGF-I=IGF-II	IGF-II	IGF-I=IGF-II	IGF-I=IGF-II	IGF-II	IGF-II
glycosylation	-	-	N-linked	N-linked	O-linked	O-linked
phosphorylation	+	-	+	-	+	?
proteolysis	-	+	+	+	+	?

* *doublet*
** *the non-glycosylated and glycosylated form respectively*
IGF-I=IGF-II *no binding preference*
? *has not yet been identified*

IGFBPs

The IGFs circulate bound to a family of binding proteins. Six IGFBPs have been characterized, designated IGFBP-1 through -6. Amino acid sequence analysis has revealed that the IGFBPs are highly homologous. They show 50-60% similarity (within species) and contain 16-18 conserved cysteines at the NH_2 and COOH terminal regions. Furthermore, they are conserved between species. They display no sequence homology to the IGF receptors.

The six IGFBPs specifically bind IGF-I and IGF-II with high affinity and specificity. Moreover, additional binding proteins with 20-30% structural homology have been described, with low affinity for the IGFs (Oh et al., 1996; Kim et al., 1997; Collet and Candy, 1998). At the cellular level IGFBPs are thought to inhibit the actions of the IGFs, but under specific circumstances they may potentiate their metabolic and mitogenic effects, as the IGFBPs are synthesized as autocrine or paracrine factors within the developing tissues in a spatial and temporally specific manner, thereby conferring functional and/or tissue specificity upon the growth regulatory apparatus. This is attained by differential post-translational modifications of the IGFBPs that can alter their biological activity. For example, phosphorylation status of IGFBPs appears to be important for their biological activity (Westwood et al., 1994). This and other post-translational processes, such as protease activity, need to be characterized further to understand the role of IGFBPs in different physiological or pathophysiological processes. Moreover, IGFBPs may

exert tissue specific effects during development independent from IGF action. Supporting this hypothesis is the evidence for the existence of specific binding of IGFBP-3 to the surface of various cell types (Oh et al.; 1993a/b). Thus, specific IGFBP receptors may exist to mediate these IGF independent effects.

Table 1 displays some characteristics of IGFBP-1 to -6. An overview of working mechanisms of the IGF system is depicted in Figure 1.

IGFBP-1

The IGFBP-1 gene is 5.2 kb long and consists of four coding exons, leading to a final transcript of 1.5 kb and a mature protein of ~30 kDa. mRNA is expressed strongly in liver and kidney and to a lesser extent in various other tissues. Regulation of IGFBP-1 gene expression is associated with metabolism and reproduction; under catabolic circumstances gene expression is strongly and acutely stimulated, e.g. in insulin-dependent diabetes mellitus (IDDM) and fasting (Lee et al., 1993; Thissen et al., 1994). Insulin and glucocorticoids appear to be the major regulators, with insulin acting inhibitory and glucocorticoids stimulatory on IGFBP-1 gene expression (Powell et al., 1991).

One of the proposed functions of IGFBP-1 is to modulate the amount of free IGF, partly because of the possibility for transcapillary transport of IGFs to the extravascular space (Bar et al., 1990; Lewitt et al., 1993). Reproduction associated functions of IGFBP-1 are seen in endometrial cycling, oocyte maturation and gestational development (Lee et al., 1993; Giudice et al., 1994; Adashi et al., 1994). Furthermore, IGFBP-1 is the predominant IGFBP in amniotic fluid, amounts fluctuating during pregnancy, whereas maternal circulatory amounts remain stable (Nonoshita et al., 1994).

Multiple studies with cell systems show that IGFBP-1 acts merely inhibitory on cell proliferation and/or cell function (reviewed in Jones and Clemmons, 1995). These inhibiting effects can be explained by IGFBP-1 interference with IGF ligand-receptor interactions, depending on the IGF-IGFBP-1 ratio. Also IGF potentiating effects of IGFBP-1 have been reported, in which IGFBP-1 is thought to provide a sustained release of IGF-I to the cells (Koistinen et al., 1990). Such potentiation may require cell surface binding of IGFBP-1. IGFBP-1 contains an integrin receptor recognition site within its primary structure. This is an Arg-Gly-Asp sequence (RGD), which allows IGFBP-1 to specifically bind to the fibronectin ($\alpha_5\beta_1$ integrin) receptor (Jones et al., 1993).

IGFBP-2

The IGFBP-2 gene is 32 kb long and consists of four exons, leading to mRNA of 2.0 kb in rat (1.6 in human). The size of the protein also differs between rat and human: 31 kDa for rat and 36 kDa in the human. Like IGFBP-1, IGFBP-2 is non-glycosylated and contains a RGD sequence. No phosphorylation isoforms have been reported. It is expressed in various tissues during development, particularly in the central nervous system, where it is for example involved in pituitary development (Kelley et al., 1996). Furthermore, it appears to be involved in transport of IGF-II to the cerebrospinal fluid. In the adult this strong expression in the central nervous system (CNS) is maintained and IGFBP-2 is the major IGFBP in cerebrospinal fluid. In addition, IGFBP-2 is expressed in a number of reproductive tissues and IGFBP-2 is the most abundant IGFBP in seminal plasma (Rosenfeld et al., 1990).

suggested
additional actions

present view

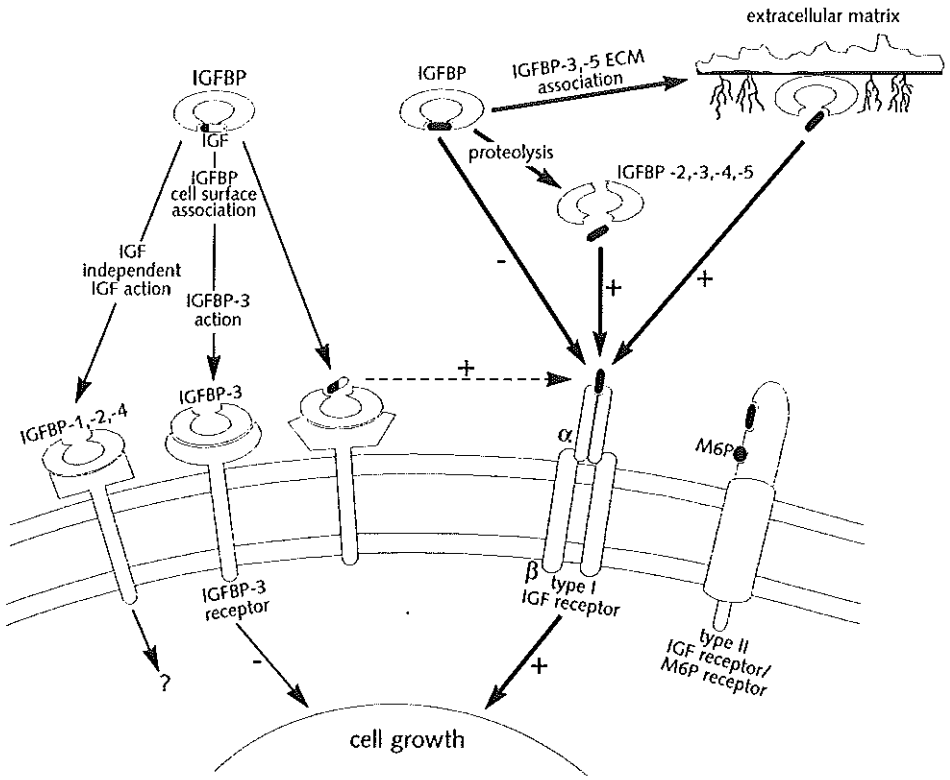
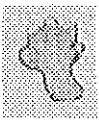


Figure 1
Schematical overview of the working mechanisms of the IGF system.



Similar to IGFBP-1, IGFBP-2 actions on IGF-mediated functions can be both inhibitory and stimulating. For its potentiating actions, cell surface association may be necessary, which indeed has been observed for IGFBP-2. Again, characterization of its physiological functions remains limited. Experiments with transgenic and knockout mice will be described in more detail in another section.

Regulation of IGFBP-2 by metabolic factors occurs similar to IGFBP-1 regulation, albeit less acutely. During insulin dependent diabetes mellitus (IDDM) growth hormone (GH) does not appear to have direct effects on IGFBP-2 expression; the reduced amount of insulin seems to be the trigger for the upregulation of IGFBP-2 (Boni-Schnetzler et al., 1990; Orlowski et al., 1990).

In porcine vascular smooth muscle cells IGFBP-2 can be cleaved by a protease in two fragments of ~25 kDa and 16 kDa (Cohick et al., 1995; Gockerman and Clemmons, 1995). IGFs are able to stimulate this proteolytic activity, IGF-II being more effective than IGF-I. In normal healthy persons fragmented forms of IGFBP-2 have been reported in circulation, due to proteolysis. However, where and why this proteolysis occurs is not clear. Very specific regulatory mechanisms are suggested to exist, correlated with extracellular matrix proteolysis (Holly et al., 1998).

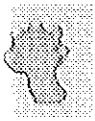
IGFBP-3

The IGFBP-3 gene has a length of 8.9 kb and encloses 5 exons. This gene is located on the same chromosome as IGFBP-1, orientated tail to tail, 20 kb apart from each other (Cubbage et al., 1990; Kou et al., 1995). The gene is transcribed into mRNA of 2.6 kb and is expressed in a large number of tissues.

High levels of IGFBP-3 mRNA are found in liver. In plasma, IGFBP-3 is complexed to IGF-I and to an acid labile subunit (ALS), that does not bind IGFs directly. The majority of IGFs in plasma is present as this ~150 kDa ternary complex (Baxter, 1988; Boisclair et al., 1998). The acid labile subunit (ALS) is expressed in liver as well as in kidney. IGF-I and ALS both are expressed in the hepatocytes, whereas IGFBP-3 mRNA is found in Kupffer cells (Chin et al., 1994; Arany et al., 1994). GH is the major physiological stimulator of IGFBP-3, but the nature of this regulation is still not understood.

IGFBP-3 exists in several modified forms; major changes include the glycosylation degree of IGFBP-3 during its cellular synthesis and its proteolytic cleavage in the extracellular environment. IGFBP-3 is heavily glycosylated and is usually produced as a doublet of 43 and 45 kDa (Baxter, 1994). Furthermore, IGFBP-3 is known to associate with the cell membrane. It appears that the potentiation of IGF action by IGFBP-3 requires its cell-surface association.

IGFBP-3 in solution is a strong competitive inhibitor of IGFs, because its affinity for IGF-I then is higher than the affinity of IGF-I for the type I IGF receptor. Upon cell association of IGFBP-3, affinity for IGF is 10-fold reduced, which means 2-fold lower than that of the type I IGF receptor (Mc Cusker et al., 1990; Conover, 1991; Binoux et al., 1991). Also by proteolysis IGFBP-3 affinity for IGF is reduced. In several cell types it has been shown that the truncated (28-30 kDa) IGFBP-3 potentiates IGF action, while intact IGFBP-3 has the opposit effect (Schmid et al., 1991; Cohen et al., 1993). Serum proteolysis is suggested to allow IGF bound to IGFBP-3 to be disseminated to other IGFBPs and/or toward the extravascular fluids and to tissues (Kelley et al., 1996). An increased rate of IGFBP-3 proteolysis may also occur to increase the availability of IGF under different physiological or pathophysiological circumstances (Holly et al., 1992; Lamson et al., 1992; Gargosky et



al., 1993; Muller et al., 1993; Fielder et al., 1994; Lee et al., 1994). IGFs seem to regulate the IGFBP-3 protease (Salahifar et al., 1997) and experiments with a non-IGFBP-3 binding IGF-I analog suggest the possibility of a direct interaction between IGF and the protease (Grimes and Hammond, 1994; Conover, 1998).

Furthermore, IGF independent inhibitory effects of IGFBP-3 have been reported (Bicsak et al., 1990; Delbe et al., 1991; Villaudy et al., 1991; Liu et al., 1992b; Oh et al., 1992; Cohen et al., 1993). For example, IGFBP-3 inhibited DNA synthesis stimulated by follicle-stimulating hormone (FSH), with or without additional IGFs (Bicsak et al., 1990). IGFBP-3 binding to the cell membrane provides a mechanism for these inhibitory effects (Oh et al., 1993a/b). It was also found that IGF binding to IGFBP-3 prevented cell membrane binding of IGFBP-3, which causes attenuation of the antiproliferative effects of IGFBP-3 (Oh et al., 1993a). This mediatory role of IGFBP-3 may also apply for other growth factors. Transforming growth factor-β (TGF-β) has antiproliferative effects, and this appears to be mediated by IGFBP-3, the expression of which is increased by TGF-β (Gucev et al., 1996).

IGFBP-4

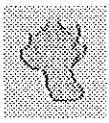
The length of the IGFBP-4 gene is 12 kb and the gene contains four exons (Gao et al., 1993). The mRNA is 2.6 kb and is expressed in a great variety of tissues, expression being highest in liver. IGFBP-4 is the smallest IGFBP and contains one glycosylation site. No membrane association has been found thus far, which suggests that the protein exists as a soluble extracellular IGFBP. IGFBP-4 on Western analysis exists as a doublet, with a non-glycosylated 24 kDa form and a glycosylated 28 kDa form.

The inhibiting actions of IGFBP-4 (Mohan et al., 1994; Rashmi et al., 1992) appear to be a result of the sequestering of local IGFs, thereby preventing receptor interaction (Cheung et al., 1991; Kiefer et al., 1992). The inhibiting effect of IGFBP-4 is lost by proteolysis. Proteolysis results in two fragments of 18 and 14 kDa, incapable of IGF binding. The IGFBP-4 protease is specific, because it is not active on other IGFBPs. Another remarkable feature is the strict dependence of the IGFBP-4 protease on IGFs for functional activity, in which IGF-II is more active than IGF-I (Conover, 1998). Interestingly, *in vitro* in various cell types, IGFBP-4 acts growth inhibiting (Cohick et al., 1993; Conover et al., 1993; Durham et al., 1995). In contrast, *in vivo* IGFBP-4 is suggested to be considered growth promoting (Rodgers et al., 1995; Pintar et al., 1997). In this way, IGFBP-4 can be seen as IGF transporter, which needs local protease activity to release IGFs.

IGFBP-5

IGFBP-5 is expressed in a large number of tissues and cell types. IGFBP-5 mRNA is most abundant in kidney, and in some cell types also smaller transcripts have been reported (Kiefer et al., 1991; Cohen et al., 1994). The protein is 29 kDa and is glycosylated (Conover and Kiefer, 1993) and phosporylation has also been reported (Jones et al., 1991). IGFBP-5 gene chromosomal location is found to be arranged in a tail to tail orientation with IGFBP-2.

IGFBP-5 is the predominant IGFBP in bone, where it promotes IGF ligand-receptor interaction by fixing IGF in the tissue. In various tissues a balance exists between the stimulating actions of IGFBP-5 and the inhibiting IGFBP-4 effects. This balance is



regulated by the IGFs and specific proteases. In contrast to IGFBP-4, IGFBP-5 is protected from proteolysis when bound to IGF. The proteolytic fragments have a reduced affinity for IGF and seem to enhance IGF-stimulated mitogenesis. Also IGF independent actions of some fragments have been reported (Andress and Birnbaum, 1992). The role of proteolysis may be to provide an alternative route for IGFBP-5 action or to inhibit IGFBP-5 stimulation of IGF action.

Furthermore, IGFBP-5 associates with the extracellular matrix (ECM), which results in protection from proteolysis. Hence, the site of ECM association may also be the cleavage site for IGFBP-5 (Clemmons et al., 1994). A mechanism has been proposed, whereby cell-associated IGFBP-5 serves as a stable IGF-binding site. As IGF affinity for cell-associated IGFBP-5 is reduced, this promotes IGF ligand-receptor interaction (Kelley et al., 1996).

In ovary, the balance between IGFBP-4 and -5 is also found and expression of both is inhibited in normal follicles. However, in degenerative, atretic follicles, expression is elevated, which might be involved in follicular selection (Adashi, 1994; Shimasaki et al., 1994). In kidney, IGFBP-5 effects appear to be growth inhibiting. For example, diabetic rat kidneys show overgrowth, accompanied by reduced IGFBP-5 mRNA levels (Kelley et al., 1995; Landau et al., 1995).

IGFBP-6

Of all the IGFBPs, least information is available on IGFBP-6. The gene is expressed as a 1.3 kb mRNA (rat), while in human two transcripts (predominant 1.1 kb and minor 2.2 kb) have been described (Kiefer et al., 1991b). The mRNA is found in a variety of tissues, with highest expression in lung and heart. The protein is 30 kDa and is glycosylated. Remarkably, the affinity of IGFBP-6 for IGF-I is lower than for IGF-II. No cell-association has been reported, neither have proteases been found. Serum levels of IGFBP-6 are lower in females and are further reduced during pregnancy. IGFBP-6 expression during mouse embryonal development is limited and restricted to late gestation (Schuller et al., 1993). In the adult mouse, expression is increased and more widespread (Schuller et al., 1994).

Localization studies of the IGF system during embryonic development.

Maternal as well as fetal IGF-I levels influence the growth of the conceptus (Gluckman et al., 1992). Fetal IGF-I is expressed at the 8 cell stage already (Rappolee, 1990), and IGF-II even at the 2 cell stage (Heyner et al., 1989; Schultz et al., 1992). The techniques used for localization studies have expanded from Northern blots to *in situ* hybridization (ISH) techniques (radioactive, non-radioactive and whole mount). Radioactive ISH is the best technique for quantification purposes. On the other hand, non- radioactive ISH is faster, less expensive and without the disadvantage of using radioactivity. When ISH is combined with quantitative RT-PCR very detailed information is obtained.

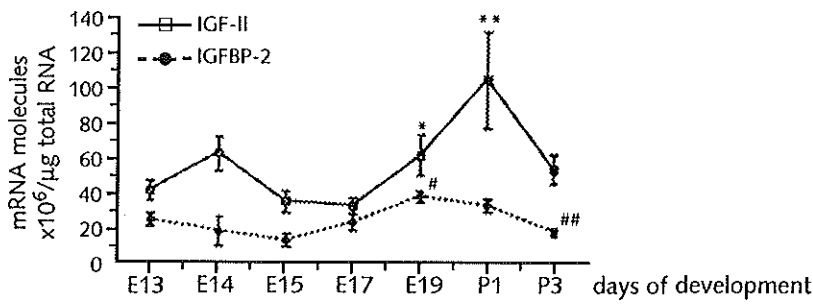
No correlation of expression of a specific set of IGF or IGFBP genes with a given stage of organ growth has been established. More detailed analysis using ISH on sections or whole mounts resulted in the identification of specific cell types expressing the genes of interest. However, until now the physiological relevance of the spatio-temporal expression of the IGF system members remains largely unknown.

The mouse kidney has been studied extensively during development (Lindenbergh-Kortleve et al., 1997; Matsell et al., 1994; Liu et al., 1993a). At 15 days post coitus (dpc),

when all stages of renal development can be recognized, IGF-I shows a scattered pattern in all cell types, while IGF-II is confined to sites of cellular differentiation. In accordance with its IGF-I and -II signal transducing role, type I receptor mRNA is expressed in all cells of the kidney. The specifically localized expression of the IGFBP mRNAs is supportive for their modulatory role in IGF actions. Strong IGFBP-2 signals are confined to differentiating cells in the branching area of the ureteric bud. IGFBP-3 is found to be exclusively located in the capillary system and expression of IGFBP-4 is located in mesenchymal cells. IGFBP-5 is found at sites of cellular differentiation, co-localizing with IGF-II. Furthermore, a close interaction in gene regulation between IGF-II and IGFBP-2 has been found, since IGF-II levels mimick the IGFBP-2 levels, which are high prenatally and decrease after a peak around birth. RT-PCR analysis revealed that in the developing kidney IGF-I and -II have an inverse quantitation profile (Fig. 2). Altogether, IGF-I and -II appear to have a distinct role at different stages of renal development. Furthermore, the overlapping or adjacent expression sites of the IGFs and their binding proteins suggest close interactions (Lindenbergh-Kortleve et al., 1997).

Radioactive ISH on developing lungs showed that the expression of IGF-I and -II is uniformly distributed in mesenchymal cells, especially those surrounding airway epithelium, from 12.5 dpc until birth (Schuller et al., 1994; Retsch-Bogart et al., 1996). In

2A



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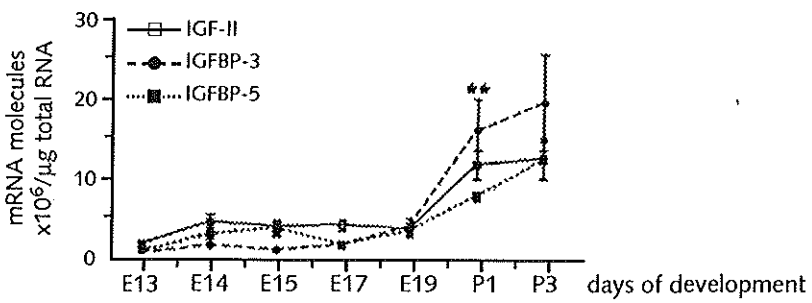
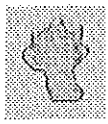


Figure 2

*(A) Quantitation profiles for mRNA of IGF-II and IGFBP-2 during mouse kidney development. Data represent the means (n=4-6) (SEM. **Significant compared with E17 (P<0.02); not significant to P3 (P<0.06). *Significant compared with E17 (P<0.03) ##Significant compared with P1 (P<0.02). #Significant compared with E17 and P3 (P<0.05)*

*(B) Quantitation profiles for mRNA of IGF-I, IGFBP-3 and -5 during mouse kidney development. Data represent the means (n=4-6) (SEM. **Significant compared with E19 for all genes (P<0.02) (reprinted with permission of the authors from Lindenbergh-Kortleve et al. (1997).*



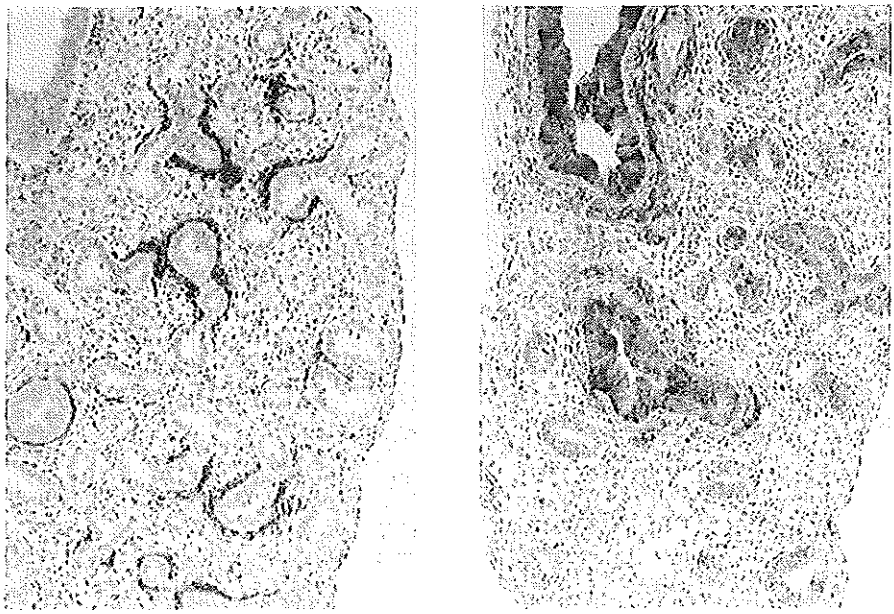
contrast, IGF-II mRNA is localized predominantly to epithelia. The overall expression of the IGF type 1 receptor at 12.5 dpc becomes restricted to the mesenchyme at 15.5 dpc. The type 2 IGF receptor is confined to the mesenchyme and medial layers of intrapulmonary vessels. The IGFs and their receptors are expressed throughout lung development with little change, mRNA of the type 2 IGF receptor being more abundant. IGFBP-2, -4 and -5 are the predominant IGFBPs in the developing lung. Throughout gestation the abundant and constant expression of IGFBP-2 is found mainly in epithelial cells, but also in mesenchyme. During all stages IGFBP-4 is expressed primarily in the mesenchyme, peaking between 16 and 19 dpc. In contrast, IGFBP-5 expression is neighbouring IGFBP-4 expression, being concentrated in the epithelial lining of the terminal bronchi and increasing as development progresses. In this way, a regulatory system, provided by the regulation of the IGFBPs, may exist to determine cell specific IGF responses during lung development. Non radioactive ISH produced similar results (Fig. 3 ; personal communication D. J. Lindenbergh-Kortleve).

The developing limb is useful to study tissue outgrowth and pattern formation. Differential and spatio-temporal mRNA expression patterns are found for the IGFs, the type 1 IGF receptor and most of the binding proteins (van Kleffens et al., 1998, Chapter 2), using whole mount ISH on limbs at 9.5 until 14.5 dpc. During early stages of limb development, IGFBP-2 and IGFBP-5 mRNA are expressed in the apical ectodermal ridge (AER) and IGF-I and IGFBP-4 in the region of the zone of polarizing activity (ZPA), a mesodermal signalling center. Limb development involves reciprocal signals between the AER and the limb mesoderm, which control the antero-posterior patterning of the limb. At 12.5 to 14.5 dpc, IGF-II, type 1 IGF receptor, and IGFBP-5 mRNA are expressed in areas where precartilage develops. Expression of the IGF system is also found in apoptotic regions, which will be discussed later. Thus, the IGF system appears to be involved in the early patterning and bone formation during limb development.

Similar localization studies have been performed for other organs and cell types during development (Schuller et al., 1993; Cerro et al., 1993; Hogg et al., 1994).

In conclusion, the IGFs, their receptors, and the IGFBPs are expressed during mouse development in a tissue-specific, spatio-temporal manner. Highest mRNA expression is found for IGFBP-2, -3, -4 and -5. IGFBP-2 and -5 mRNA are mainly found in epithelial structures, although also mesenchymal localization has been demonstrated. In contrast, IGFBP-4 mRNA is expressed merely in mesenchyme. The IGFs and their receptors are expressed more generally, pointing to cell-specific modulation of IGF-action by the IGFBPs. Whether mRNA expression equals protein localization, is at present unresolved. It is well known that there are several posttranslational regulatory modifications. In addition, secretion and trapping by (extra) cellular moieties may result in a different immunohistochemical picture and would put the physiological significance of these proteins in another perspective.

Moreover, it is of specific interest that, for instance in the limb, the location of expression of genes encoding proteins of the IGF system corresponds with the expression of other genes involved in limb development. These include genes encoding growth factors, such as fibroblast growth factor (FGF-8), growth and differentiation factor (GDF-5), the TGF-β family members bone morphogenetic proteins (BMP-2 and BMP-4), and more general developmental genes, such as Sonic hedgehog (SHH), and genes regulated by retinoic acid (RA) (van Kleffens et al. 1998; Chapter 2). Further studies are required to prove a functional interrelationship.



3A 3B

Figure 3
ISH (non-radioactive) on fetal mouse lung (15 dpc).
(A) IGF-I mRNA is localized in mesenchymal cells adjacent to bronchial epithelium
(B) IGFBP-5 mRNA is found in both epithelial lining of bronchi and mesenchymal cells.

Localization of the IGF system in the placenta

Cell invasiveness, and cell proliferation and differentiation are processes during placental development dynamically controlled by many growth factors and proto-oncogene products. It is not without reason that this very complex process has been termed a "pseudomalignant" process (Kirby and Cowell, 1968). The IGF system has been implicated as one of the growth factor systems contributing to the normal placental growth in rodents. Most compelling evidence is that in mice with an IGF-II gene deletion placental development is arrested (DeChiara, 1990). The placenta is smaller as a result of a much lower number of glycogen producing cells (Lopez et al., 1996).

The preimplantation embryo is bathed in fluid that is a rich source of IGFs and their binding proteins. IGF-I is most predominantly synthesized in glandular cells and epithelium of the uterus, reaching high levels just around initiation of implantation (Kapur et al., 1992). IGFBP-1 is strongly expressed in preimplantation endometrial glands. However, IGFBP-2 while being expressed markedly during proestrus (Girvigian et al., 1994) is not demonstrable in the pregnant uterine luminal epithelial cells (Cerrro and Pintar, 1997). The shift of IGFBP-1 and 2 expression may be a critical step in implantation of the blastocyst (Cerro and Pintar, 1997).

Following implantation during the period of rapid decidual proliferation, IGFBP-3,-4 and -5 are increasingly expressed in a laminar array at the boundary between decidua and nondecidualized endometrium, whereas IGF-II gene expression is abundant in the fetal

trophoblast tissues. Type 1 and 2 IGF receptor colocalize with IGF-II gene expression, suggesting that both receptors compete for IGF-II. In the decidual vasculature IGFBP-3 and -4 expression is found in dilating blood vessels.

Several IGFBP mRNAs are detected in the uterine myometrium. As decidualization proceeds, myometrial IGFBP-2 and -6 expression increases, while IGFBP-5 expression decreases.

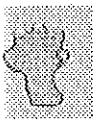
In mid- and late gestation, decidualization is complete, placental structures are well differentiated, and little change in expression of the IGF system is noted (Cerro and Pintar, 1997).

Thus, the uterine pattern of IGFBP expression is consistent with regulating (fetal) trophoblast development and regulating (maternal) decidual exposure to IGFs. Zhou and Bondy (1992) suggest that in view of the specific spatio-temporal pattern of IGF-II and IGFBP-2 expression, there may be a significant functional relationship betweeen IGF-II and IGFBP-2 in the placenta. During the early invasive period when the placenta is established, IGF-II expression is high and unopposed by IGFBP-2 ,whereas near term this ratio is reversed (Zhou and Bondy, 1992).

Results from the transgenic and targeted gene inactivation approach

The advantage of creating transgenic and knockout mouse models is that synthesis of one gene product is changed via overexpression, ectopical expression, or null mutation (knockout) and that the effects on the whole organism can be studied. It has been well established that both IGF-I and IGF-II are important for prenatal growth, together with the type 1 IGF receptor. This replaces the prevailing idea that IGF-II is the major mediator of fetal growth. IGF-II knockout mice showed a reduction in prenatal growth and a birth weight of 60% of normal (DeChiara et al., 1990). However, inactivation of the IGF-I gene also resulted in a similar reduction of birth weight and caused neonatal lethality in most mice (Liu et al., 1993b). Null mutants for the type 1 IGF receptor were even more reduced in birthweight (45% of normal) and all mice died shortly after birth (Liu et al., 1993b). These results suggest that the type 1 IGF receptor (IGF1R) serves as a receptor for both IGF-I and IGF-II in fetal development. When the type 2 IGF receptor (IGF2R) is deleted, the resulting mice are 25-30% larger than normal and have elevated levels of circulating IGF-II and IGFBPs (Lau et al., 1994). The occurring perinatal lethality is caused by cardiac abnormalities, including overgrowth of the ventricular myocardium and septal and valvular defects. This reveals the important role for the type 2 IGF receptor during development, probably through binding and removing IGF-II from the circulation (Czech, 1989).

Surprisingly, mice with combinations of various gene deletions such as the IGF1R/IGF2R double mutant, develop normally, pointing to the existence of a third IGF receptor, presumably being the insulin receptor (Accili, 1997). This receptor should preferentially bind IGF-II, since IGF1R/IGF2R/IGF-II mutants are nonviable dwarfs (Ludwig et al., 1996). The gene targeting experiments have also revealed that the IGF2R and IGF-II genes are imprinted. Only paternal transmission of the mutated IGF-II gene leads to the growth deficient phenotype in the heterozygote IGF-II (p-). This phenotype is identical with the homozygote IGF-II(-/-) (DeChiara et al., 1990). Likewise, the IGF2R gene is maternally imprinted (Barlow et al. 1991).



Of the IGFBPs, thus far null mutants of IGFBP-2 and -4 and -6 have been described (Pintar et al., 1995; Pintar et al., 1998). The IGFBP-2 mutant has a decreased spleen size and increased circulating levels of IGFBP-1, -3, and -4. However, since IGFBP-2 is present abundantly in several tissues during development, a more dramatic phenotype was expected. Disruption of the IGFBP-4 gene leads to a relatively lower postnatal weight (10-15%) (Pintar et al., 1998). This is in contrast with the cell growth inhibiting function of IGFBP-4 *in vitro*. However, as mentioned before, *in vivo* results point to a stimulatory role for IGFBP-4 in growth, which is confirmed by the knockout experiment. The IGFBP-6 gene deletion does not appear to result in prenatal lethality. Although no detailed growth curves are available yet, the IGFBP-6 knockout mice do not appear to exhibit gross defects in growth (Pintar et al., 1998). This lack of dramatic changes points to a functional take over by other IGFBPs. However, the effects of gene disruption of the remaining IGFBPs and the construction of mice in which more than one gene of the IGF system has been deleted might give further clues.

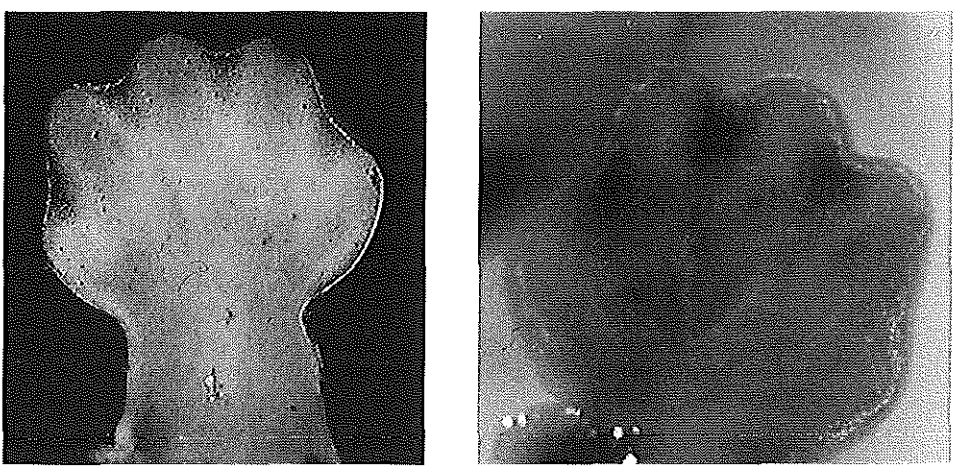
Transgenic animals that overexpress a gene or that have ectopical expression have been created for IGF-I and -II and IGFBP-1 and -3. As expected, mice overexpressing IGF-I at 1.5 times normal serum values were growth-enhanced, which was manifested by a 1.3-fold increase in weight as a result of selective organomegaly, including spleen, pancreas, kidney and brain (Matthews et al., 1988). This animal model revealed that IGF-I has growth promoting actions separate from GH (Behringer et al., 1990). Furthermore, it was shown that IGF-I can induce IGFBP-3 and -2, independently of GH. IGF-II transgenic mice with circulating levels of IGF-II 2-3 times higher than those of controls, had negatively correlating IGF-I levels and positively correlating IGFBP-2 levels (Wolf et al., 1994). This suggests that displacement of IGF-I by IGF-II occurs, and that IGF-II might be a major regulator of IGFBP-2. Body growth of these transgenes was not altered, and only subtle time-specific effects on organ growth were noted, such as enlarged kidneys (Wolf et al. 1994). However, when the transgene was expressed in the mammary gland, mammary tumors developed in all mice (Bates et al., 1995). When the same gene was controlled by a keratin promoter, overgrowth of skin resulted, but no tumors were formed (Ward et al., 1994).

IGFBP-1 and IGFBP-3 have been overexpressed in transgenic animals, using different promoters. Depending on the promoter used and the number of transgenes incorporated, a modest decrease of birth weight and a reduced brain weight, or no effect at all, was observed (Murphy et al., 1993; 1995; Dai et al., 1994; Rajkumar et al., 1995; 1996).

It can be concluded from these studies in genetically manipulated animals that the IGF system plays an important role during development. The IGFs are growth promoting, while at least IGFBP-1 and probably IGFBP-3 serve to fine-tune IGF actions. However, one of the drawbacks of elucidating gene function by creating transgenic animals is that the results are dependent on the transgene and the promoter used. Also genetic background of the animals used in gene manipulation experiments influences the phenotypical changes. Thus, research needs to be focused on these "internal" factors, to elucidate the reasons for the background dependent effects of gene manipulation. The results of knock-out studies of some of the genes of the IGFBP family so far reported, suggest functional redundancy of different IGFBPs. In addition to evolutionary explanations, there may be functional aspects that may not have become evident. An apparently redundant gene might very well increase fitness, not only as a back-up gene, but also in more subtle ways (Cooke et al., 1997).

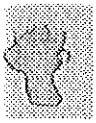
Apoptosis

During embryogenesis many morphological changes of tissue take place. Often apoptosis is involved, allowing remodelling of certain embryological structures. Apoptosis or programmed cell death is a physiological type of cell death. It is characterized by distinct biochemical and morphological changes, such as DNA fragmentation, plasma membrane blebbing, and cell volume shrinkage. IGF-I is one of a variety of survival factors (among other growth factors, such as EGF, NGF, PDGF) preventing the onset of apoptosis. The inhibitory effect of IGF-I on apoptosis appears to be mediated by the type 1 IGF receptor (Resnicoff et al., 1995). The role of the IGFBPs in these apoptosis inhibiting effects of the IGFs and the type 1 IGF receptor is suggested in several *in vitro* studies (Phillips et al., 1994; Guenette et al., 1995; Chun et al., 1994; Tonner et al., 1995). The involvement of IGFs, their receptors and binding proteins in apoptosis taking place during embryogenesis, so far has only been described for developing mouse limbs (van Kleffens et al., 1998; Chapter 2). In limb, IGF-I and IGFBP-2, -3, -4, and -5 mRNA expression is specifically found in regions where apoptotic cells, indirectly identified by Nile Blue are observed. Around 13.5 dpc apoptosis occurs in the interdigital zones to attain the final morphology of the digits. During that time, in these areas IGF-I and IGFBP-2 to -5 are expressed at different sites, partially overlapping. IGFBP-2, -4 and -5 are localized in the interdigital zone, while IGF-I and IGFBP-3 expression borders this region. IGFBP-2 expression covers the whole interdigital region, while IGFBP-5 is found in the proximal interdigital zone, expanding to the whole area at 14.5 dpc (an example is given in Fig. 4). IGFBP-4, however, remains restricted predominantly to the proximal interdigital zone. Furthermore, IGFBP-3, -4, and -5 are found in phalangeal joint areas, where apoptosis causes the formation of the joints. We speculate that specific members of the IGF system mediate apoptotis in the interdigital zone and in the joint areas in a spatio-temporal manner. More research should be undertaken to functionally proof the regulatory role of the IGF system in developmental apoptosis.



4A 4B

Figure 4
(A) Nile Blue staining of apoptotic cells in the interdigital zones of a 13.5 dpc mouse limb
(B) Expression pattern of IGFBP-2 mRNA as determined by whole mount ISH (non-radioactive) of a 13.5 dpc mouse limb.

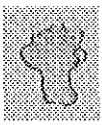


Scope of this thesis

Insulin-like growth factors (IGFs) promote cellular mitosis and differentiation and have been implicated in fetal and placental growth. Despite all the research of the past years that has been focused on unraveling the mechanisms of action during embryonal development of the IGF system and the IGFBPs in particular, still large gaps in our understanding remain. This is due in part because of the multiple actions of the IGFBPs. They can both inhibit as well as stimulate IGF action, and also IGF independent actions have been described.

Therefore, this thesis focuses on the role of the IGF system during growth and differentiation, on the mRNA and protein levels, during mouse development.

Chapter 2 describes the mRNA expression patterns of the IGF system in the developing mouse limb, a useful model to study tissue outgrowth and pattern formation. To extend the obtained mRNA data we generated polyclonal antibodies specifically directed against mouse IGFBP-1 through -6, which is shown in Chapter 3. In that chapter, the methodology is described, and a comparison is made between IGFBP protein and mRNA localization in mouse embryonal sections. The possible involvement of the IGF system with developmental apoptosis is described in Chapter 4. That chapter deals with an extensive study on the developmental IGF system expression patterns of the mouse, in which mRNA expression patterns are compared with protein localization of the IGF system. Furthermore, the colocalization of specific IGF system members with regions with apoptosis is investigated. To further investigate the physiological role, *in vivo*, of one of the IGFBPs, the effects of increased circulating levels of IGFBP-1 in relation to IGF-I and GH in the adult Snell dwarf mouse were analyzed, which resulted in Chapters 5 and 6. In Chapter 5, the effects of administration on normal body and organ growth are described. Chapter 6 focuses on specific changes of IGF system mRNA and protein localization in the kidney after administration. Finally, the results of the studies described in this thesis and future directions of research are discussed in Chapter 7 and summarized in Chapter 8.



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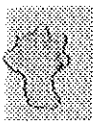

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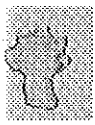

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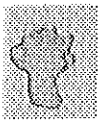

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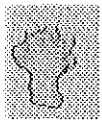

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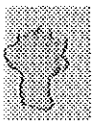

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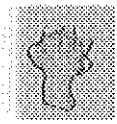

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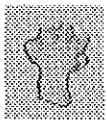

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General introduction

Chapter 2

mRNA expression patterns of the IGF system during mouse limb bud development, determined by whole mount *in situ* hybridization

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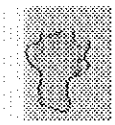


Abstract

During limb development the primary limb bud requires various signals to differentiate. IGF-I and -II serve as ubiquitous cellular growth promoters and are modulated by their binding proteins (IGFBPs), which inhibit or augment IGF bioavailability. This is the first study to give a complete overview of the mRNA expression patterns of IGF-I, IGF-II, type 1 IGF receptor (IGF-IR) and six IGF binding proteins (IGFBP-1 to -6) in embryonic mouse limbs, at various stages of development, by whole mount *in situ* hybridization.

Our results show that all the members of the IGF system, except IGFBP-1 and -6, have specific spatio-temporal mRNA expression patterns. IGFBP-2 and -5 are found in the apical ectodermal ridge (AER), and IGF-I and IGFBP-4 in the region of the zone of polarizing activity (ZPA). IGF-II and IGF-IR are found in regions of precartilage formation. At 13.5 dpc the IGF system colocalizes with apoptosis areas; IGFBP-2, -4 and -5 are found in the interdigital zone, while IGFBP-3 and IGF-I border this region. Furthermore, IGFBP-3, -4 and -5 are found in the phalangeal joint areas, at an early stage of joint formation. This supports the hypothesis that the IGF system may be involved in chondrogenic differentiation of mesenchyme and the regulation of apoptosis in the developing limb.

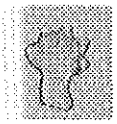
Introduction

The embryonic developing vertebrate limb is a useful model to study tissue outgrowth and pattern formation. During the first stages of limb development a bud is formed, which obtains patterning along all three axes (antero-posterior, a-p; dorso-ventral, d-v; proximo-distal, p-d). This process is influenced by the apical ectodermal ridge (AER). The AER promotes outgrowth and has anti- differentiative effects on the subridge mesoderm (Zwilling, 1955). When the AER is removed in an early stage, limb bud cells stop dividing and the entire limb bud fails to develop (Summerbell, 1974). Limb development involves reciprocal signals between the AER and the limb mesoderm, which contains several important signalling regions. The area underlying the AER is known as the progress zone (PZ). The cells in the PZ are also influenced by patterning signals from the zone of polarizing activity (ZPA), a region of mesoderm localized at the limb bud posterior margin (Crossley et al., 1996). Together, signals from these regions contribute to the regulation of limb bud outgrowth and patterning (Tickle, 1996).

Members of the Insulin-like growth factor (IGF) system are likely to be involved in limb bud development (Dealy and Kosher, 1995). The IGFs (IGF-I and IGF-II) are single chain polypeptides mediating many of the growth promoting effects of growth hormone (Daughaday et al., 1972). The *Igfs* are expressed in a wide variety of cell types where they serve as ubiquitous cellular growth promoters. Effects of both IGF-I and IGF-II are mediated through the type-I IGF receptor. The type-II IGF receptor, identical to the cation independent mannose-6-phosphate receptor (MP6R), has no known growth mediating effects (reviewed by Jones and Clemmons, 1995). However, when the mouse M6PR gene is mutated, a larger mouse results (Wang et al., 1994; Lau et al., 1994). In the circulation and throughout the extracellular space IGFs are bound to the IGF binding proteins (IGFBPs), with affinities comparable with those for the IGF receptors. Therefore, the IGFBPs, a family of six related proteins (Drop et al., 1992), are believed to modulate IGF actions either by inhibiting or augmenting IGF bioavailability (review Jones and Clemmons, 1995). Even direct, receptor mediated effects, independent of the IGFs, have been suggested (Liu et al., 1992; Oh et al., 1993).

It has been shown that production of IGF-I in the mesonephros has inductive effects of mesodermal condensation in the lateral plate during early limb development (Geduspan and Solursh, 1993). Furthermore, IGF-I induces the formation of limb bud-like structures *in vivo* from prospective limb regions (Dealy and Kosher, 1996). Maintenance of IGF-I expression by the subridge mesoderm is dependent on the AER, while the ability of fibroblast growth factors (FGFs) to stimulate outgrowth and proliferation of the subridge mesoderm is dependent on IGF-I activity (Dealy et al., 1996). Another prominent aspect in limb development is the condensation of mesoderm to form precartilage.The extent of chondrogenesis in a limb bud cell culture system can be affected by culture conditions and additions of various growth factors (Schofield and Wolpert; 1990). Also, studies indicate that the IGFs and their binding proteins play a role in bone remodelling (reviewed by Rosen et al.,1994). Additionally, IGF-I, IGF-II, IGF-IR and IGFBP-2 mRNA expression (Streck et al., 1992; Geduspan et al., 1992) show distinctive patterns in limb during embryogenesis. Taken together, this suggests that the IGF system is involved in limb development.

After the onset of precartilage formation the morphology of the digits has to be established, in which apoptotic cell death is a prominent aspect (Hurle et al., 1996).



Although several genes have been described to be expressed in the interdigital zone during the apoptotic events, it remains to be resolved which genes are involved in apoptotic cell death (Hurle et al., 1996). IGF-I, IGF-II and the type I IGF receptor have an inhibitory effect on apoptosis (Resnicoff et al., 1995; Ueda and Ganem, 1996). In hepatocytes IGF-II blocks apoptosis triggered by deregulated N-MYC expression (Ueda and Ganem, 1996) and IGF-II suppresses apoptosis during oncogenesis *in vivo* (Christofori et al., 1994). The effect of the type I IGF receptor is even stronger *in vivo* than *in vitro*, in several transplantable tumors (Resnicoff et al., 1995). It has been proposed that IGFBP-3 blocks the suppressive effect of human chorionic gonadotropin (hCG) on apoptosis by neutralizing IGF-I (Chun et al., 1994). Finally, IGFBP-5 may serve to inhibit IGF-I mediated cell survival in the involuting mammary gland (Tonner et al., 1995).

In the present study, we are the first to obtain a complete overview of the localization of the IGF system during various stages of mouse limb development. In order to determine whether the IGF system colocalizes with mesenchymal differentiation and apoptosis during limb formation we analysed the mRNA expression patterns of IGF-I, IGF-II, IGF-IR and IGFBP-1 to -6 in 9.5-14.5 dpc mouse limbs by non-radioactive whole mount *in situ* hybridization. Furthermore, we stained areas of cell death in the developing limb with Nile blue sulfate.

Materials and methods

Mouse embryos

Pregnant Balb/c mice were sacrificed, for whole mount ISH or Nile blue sulfate staining. The time of conception was defined by the presence of a vaginal plug the morning after mating, being designated day 0.5 of embryonic development. Embryos were isolated, directly fixed in 4% paraformaldehyde O/N, staged according to Theiler (1972) and stored in 70% ethanol at -20°C. As limb bud formation starts at 9.5 days post coitum (dpc) and separation of the digits is almost complete at 14.5 dpc this time span was choosen in our study (Theiler stages 15 to 22). For each probe and timepoint n=5 is used.

Dig-RNA probes

Sense and anti-sense RNA probes for IGFBP-1 to -6, IGF-I, IGF-II and IGF-IR were obtained following the manufacturers procedures (Boehringer Mannheim GmbH, Mannheim, Germany). Mouse IGFBP-1 to -6 cDNA fragments corresponding to respective amino acid positions 1-163, 98-225, 110-178, 110-186, 88-182 and 84-140, an *Eco*RI IGF-I fragment and a *Bam*HI/*Sac*I IGF-II fragment were ligated into pTZ18 (sense probes) and pTZ19 (antisense probes) (Pharmacia, Stockholm, Sweden). cDNAs encoding mouse IGF-I and -II were kindly provided by Dr. G. I. Bell (Howard Hughes Institute, Chicago, IL, USA). A 265 bp *Eco*RI/*Sma*I fragment of the rat IGF-IR was kindly provided by Dr. H. Werner and Dr. D. LeRoith (National Institutes of Health, Bethesda, MD, USA). Testing of the transcripts was done by agarose gel electrophoresis, blotting, incubation with an alkalic phosphatase conjugated anti-DIG antibody and staining with NBT (4-nitroblue tetrazolium chloride) and BCIP (5-bromo-4chloro-3-indolyl-phosphate; Boehringer Mannheim GmbH, Mannheim, Germany).

Whole Mount In Situ Hybridization

The procedure as described previously (Barth and Ivarie, 1994) was optimized for this experiment. Embryos were washed with PBST and treated with proteinase K (10 (µg/ml) for 15 minutes at room temperature. After 2 hours of prehybridization embryos were hybridized (250 ng/ml RNA probe) during 36 hours at 60°C in a hybridization mixture containing 50 % formamide, 4xSSC, 1xDenhardts, hering sperm DNA (0.5 mg/ml), yeast t-RNA (0.25 mg/ml), 10% dextran sulfate and heparine (50 (µg/ml). Then the embryos were washed O/N at 60°C and blocking was performed, before adding anti-DIG (1:2000). Detection took place by adding detection buffer containing 100mM Tris-HCl (pH 9.5), 150 mM NaCl, 25 mM $MgCl_2$ and 0.3% Triton, followed by 2-6 hours incubation in the same buffer with 1 mM levamisol, 10% PVA (polyvinylalcohol, MW 31-50 kD, Aldrich Chemical Co. Milwaukee, WIS, USA), BCIP and NBT. The reaction was stopped by washing with TE and stained embryos were stored at 4°C in TE.

Nile Blue staining

To detect areas of cell death in the developing limb, embryos were stained with Nile Blue A (Sigma-Aldrich, The Netherlands), a lysosomotropic vital dye. The number of granules stained blue by this dye corresponds to the number of clusters of dead cells phagocytized by macrophages (Kimura and Shiota, 1996). A water-saturated solution was diluted 1:500 in water. Vital embryos varying from 12.5 to 14.5 dpc were stained for 1 hour at room temperature. After washing 5 times in water limbs were analysed directly or left in 4°C in PBS for one night.

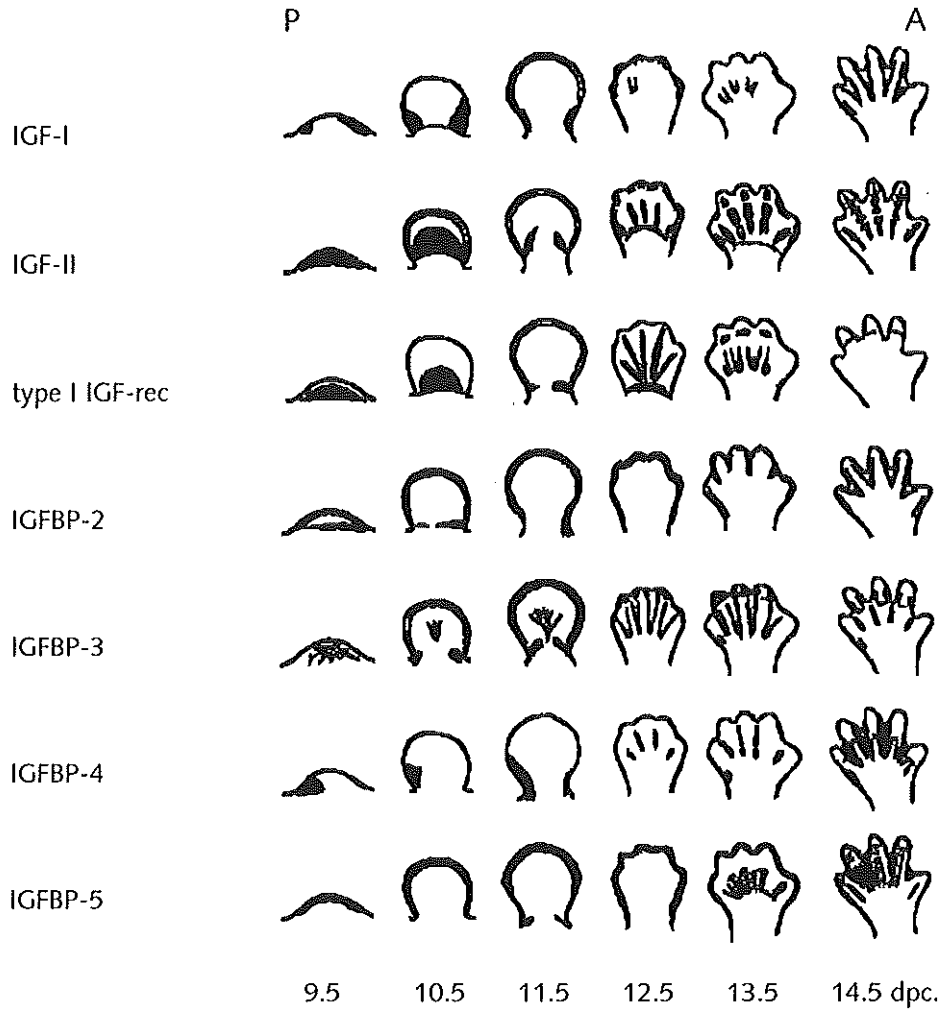


Figure 1
Schematical overview of whole mount in situ hybridizations of mouse limbs (9.5-14.5 dpc), showing mRNA expression patterns of IGF-I, -II, IGF-IR, IGFBP-2 , -3, -4 and -5, in fore limbs only. A = anterior; P = posterior. mRNA expression patterns of Igf-1 and IGFBP-4 show some overlap at early stages (9.5-11.5 dpc) in the ZPA region and anterior mesoderm. IGFBP-2 overlaps with IGF-I and IGFBP-4 in a lesser extend. At these early stages also IGF-II and IGF-IR show corresponding expression patterns, in the proximal mesoderm. Furthermore, two mesodermal regions, anterior and posterior, are found to contain IGFBP-3 mRNA. These expression regions increase at 11.5 dpc, resembling IGF-IR and IGFBP-5 expression in this region at the same time point, and it also falls within the expression domain of IGF-II at 11.5 dpc. At later stages of limb bud development overlap is found in the AER for IGFBP-2 and IGFBP-5 at 11.5 dpc. In the interdigital zones expression is shown of IGF-I, IGF-IR, IGFBP-2, -3, -4 and -5 However, coexpression is only seen for IGF-IR, IGFBP-3, -4 and -5 at 13.5 dpc in the proximal interdigital region and for IGFBP-2 and -5 in the distal interdigital zone at 14.5 dpc. Colocalization of IGF-I and IGFBP-2 is found at 14.5 dpc in part of the thin mesodermal cell layer surrounding the future phalanges.

Results

The expression patterns of the members of the IGF system, obtained by non-radioactive whole mount *in situ* hybridization of developing limb buds, are summarized in Figure 1. Expression patterns were analysed in whole limbs until 11.5 dpc. During later stages analysis was focused at the specific expression patterns in the hand and foot. It is important to mention that the development of the hind limb lags behind the fore limb. Taking this delay into account both fore limbs and hind limbs show comparable signals for all probes. The developmental stages mentioned here apply to fore limb development.

No signal was found for hybridizations of 9.5 - 14.5 dpc limbs with IGFBP-1 (data not shown). This is in concordance with the existing data for IGFBP-1 expression levels in mouse during embryonic development, being low, except in liver. For the same reason no IGFBP-6 hybridization signal could be detected in limb during development.

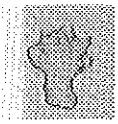
Nile blue staining

Nile blue sulfate staining of mouse limbs of 12.5, 13.5 and 14.5 dpc show the changing patterns of cell death during development (Fig. 2A-C). Figure 2A shows a 12.5 dpc limb, where areas of cell death colocalize with the anterior and posterior proximal parts of the ectoderm. At 13.5 dpc the whole ectoderm contains regions with cell death. At 13.5 dpc the interdigital staining is maximal, being more intense in the interdigital zones I and IV than II and III. At this stage also staining of metacarpal-phalangeal joints and between metacarpals is observed (Fig. 2B). At 14 dpc the latter signal is most intense, while also the phalangeal joints are stained. At 14.5 dpc only the part of the ectoderm surrounding the basal membrane is stained (Fig. 2C).

Negative controls for the *in situ* hybridization experiments consisted of whole mount *in situ* hybridizations of limbs at various time points with a sense probe of the gene of interest. None of these sense probes gave any signal, as is shown for a 14.5 dpc limb (Fig. 3).

IGF-I

Both in 9.5 dpc and 10.5 dpc limb buds strong IGF-I signals are found in the anterior mesodermal cells. Expression is also visible in posterior mesodermal cells, in the region where the ZPA is located (Fig. 4A). At 11.5 dpc these expression regions become smaller and at this stage the prominent marginal vein, located just subjacent to the AER is stained (Fig. 1; Fig. 4B). At 12.5 dpc the interdigital region is stained locally, v-shaped and strongest proximally, starting between digit III and IV (Fig. 1). At 13.5 dpc, the same signal is also found between digits II and III and between IV and V. At 14.5 dpc the interdigital signal is still located at the borders of the interdigital region, extending more distally than at 13.5 dpc (Fig. 4C). Furthermore, expression is found in a small posterior proximal part of the hand/foot.



IGF-II

At 9.5 dpc IGF-II mRNA expression is found in mesodermal cells through the whole limb bud. At 10.5 dpc the expression domain becomes limited to the proximal mesoderm, forming a ring between the hand/foot and arm/leg (Fig. 4D). This signal sustains until 12.5 dpc. Hybridization signal in the prominent marginal vein is found at 10.5 dpc and 11.5 dpc (Fig. 4D), which has almost disappeared at 13.5 dpc (Fig. 4E). At 12.5 dpc mRNA expression of IGF-II appears in the first areas where pre cartilage is formed (Fig. 1). At 14.5 dpc bone primordia of the digits show signals (Fig. 4F). It is clearly visible that the formation of the individual phalanges already has taken place at this stage. IGF-II signal at 14.5 dpc is highest in the cells surrounding the phalangeal joint regions.

IGF-IR

Hybridizations with the type I IGF receptor gave faint signals. Expression patterns during early stages overlap with IGF-II expression in the developing limb. At 9.5 and 10.5 dpc mesodermal cells express IGF-IR, being localized through the whole mesoderm at 9.5 dpc, while at 10.5 dpc the expression becomes localized to the proximal mesoderm. At 11.5 dpc we found expression in the anterior and posterior proximal mesoderm, being stronger in the anterior part (Fig. 4G). Furthermore, expression is found in the marginal vein, albeit at a very low level (Fig. 4G). At 12.5 dpc expression occurs in the region where the handplate forms and in the prechondral skeleton of the digits (Fig. 4H). At 13.5 dpc signal is found more specifically surrounding areas of chondrogenesis; separate digit anlagen show expression, albeit at a very low level (Fig. 4I). Furthermore, signal is found in pre-phalangeal jointareas and in the proximal interdigital regions. At 14.5 dpc the overall expression is still low and is seen only in part of the cartilage at the distal part of the hand/foot (Fig. 1).

IGFBP-2

At 9.5 dpc signal is located at the sites where the limb bud appears, being most intense anteriorly (Fig. 5A). At 11.5 dpc the signal is still localized anteriorly and also in the marginal vein. At 9.5 dpc a faint signal is found in the apical ectodermal ridge (AER), which becomes more intense at 11.5 dpc. This ectodermal signal remains intense until 12.5 dpc and fades away at 13.5 dpc. At this time point signal in the disappearing marginal vein is also seen. Furthermore, an interdigital, mesodermal signal appears at 12.5 dpc, which is gradually becoming stronger towards the distal ectoderm and shows a sequential expression pattern, starting between digit III and IV (Fig. 5B). At 14.5 dpc expression is found in the proximal interdigital regions. Signal also becomes located in the mesoderm surrounding the skeletal primordia, presumably being bloodvessels, and still most intense distally, in the region where the basal membrane is located (Fig. 5C).

IGFBP-3

At 9.5 dpc to 11.5 dpc signal is found in the middle part of the limb bud, presumably in developing bloodvessels (Fig. 5D). At 10.5 dpc the marginal vein, underlying the ectoderm, is also stained. Furthermore, signal is found in the posterior and anterior proximal mesoderm, strongest anteriorly. The 11.5 dpc limb bud shows similar patterns (Fig. 5D). At 12.5 dpc signals are seen in the interdigital regions, but only at the most proximal sites and in a small part of the mesodermal cell layer bordering the interdigital

region. These resemble the IGFBP-2 signals and are presumably bloodvessels (Fig. 5E). One day later similar signals are found, together with the staining of distal mesodermal cells, bordering the region where separation of the digits already has taken place. At 14.5 dpc the proximal interdigital expression region is enlarged in the distal direction. A thin mesodermal cell layer located at the borders of the interdigital zone and in the future joints of the phalanges also show expression of IGFBP-3 mRNA (Fig. 5F). Again a sequential expression pattern is found for IGFBP-3, in the developing joints (compare IGF-I and IGFBP-2, being expressed sequentially in the interdigital zone). Furthermore, expression is found in a posterior proximal region (Fig. 5F).

IGFBP-4

At 9.5 dpc limbs show expression of IGFBP-4 in the posterior proximal mesoderm, or ZPA region. At 10.5 dpc signal is also located in the posterior proximal mesoderm, but in a smaller region (Fig. 5G). One day later both posterior and anterior mesodermal cell layers are stained, the posterior signal being most intense. At 12.5 dpc IGFBP-4 signal is found in the proximal mesoderm, in the interdigital zone. This interdigital signal is becoming apparent at 12.5 dpc, starting between digits III and IV, showing the already mentioned sequential expression pattern (Fig. 5H). At 13.5 dpc the overall interdigital signal has decreased, but a strong expression is located in a small most proximal interdigital region. At 14.5 dpc the interdigital signal has become located towards the borders of the interdigital zone, in mesenchymal cells surrounding the proximal phalangeal bone primordia. IGFBP-4 is also expressed in a small posterior proximal region, comparable with IGF-I and IGFBP-3. Furthermore, signals are found between the developing two proximal phalanges, in the future joints (Fig. 5I).

IGFBP-5

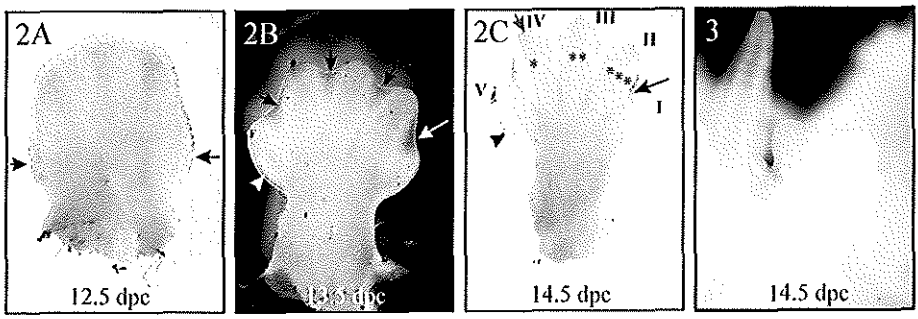
During all stages of limb development IGFBP-5 mRNA is expressed in the ectoderm. At 9.5 dpc and 11.5 dpc an ectodermal signal is seen, also including the AER (Fig. 5J). Furthermore, a weak signal is obtained, at 11.5 dpc, in the border region between paddle and arm, presumably being muscle primordia. These signals are similar at 12.5 dpc, while at 13.5 dpc, when separation of the digits becomes evident, additional signals appear. The interdigital area and specific proximal regions in the cartilage of the developing digit bones are stained, which is, again, a sequential event (Fig. 5K). In the 14.5 dpc limb bud a thin region bordering the interdigital zone contains an IGFBP-5 signal gradient, with strongest signal proximally and also the borders of the bone primordia are stained, albeit less intense. Furthermore expression of IGFBP-5 mRNA in the future phalangeal joints is seen (Fig. 5L). Similar signals are found at 13.5 dpc between the two proximal pre phalanges (Fig. 1).

Figure 2
Nile blue sulfate staining of whole mouse limbs (12.5-14.5 dpc) to detect regions with cell death.
*Cell death regions in a 12.5 dpc hindlimb are restricted to posterior and anterior zones (arrows) (A). In a 13.5 dpc forelimb cell death occurs in the interdigital zones (arrows) and in a posterior proximal region (arrowhead)(B). The cell death patterns in a 14.5 dpc forelimb are comparable to the 13.5 dpc patterns, but also include staining in the prephalangeal joint regions (asterixes), and between the metacarpals. The occurrence of cell death in the pre phalangeal joint areas is sequential, starting in digit IV (sequence indicated by *, ** and ***)(C).*

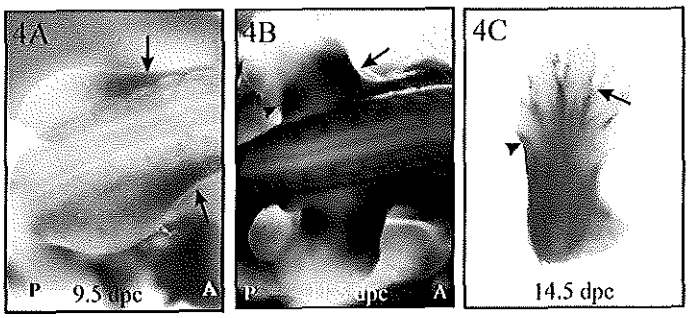
Figure 3
Negative control. Whole mount in situ hybridization of a 14.5 dpc limb, using an Igfbp-2 sense probe. As is expected no signal is found.

Figure 4
Distribution patterns of mRNA of IGF-I, -II and IGF-IR in limbs of 9.5-14.5 dpc (A-I). IGF-I in a 9.5 dpc hind limb is localized primarily in the anterior mesoderm (arrows) (A). Expression of IGF-I in a 11.5 dpc hind limb in both anterior (arrow) and posterior (arrowhead) mesodermal cells (B). IGF-I expression in a 14.5 dpc fore limb at the borders of the pre-cartilage (arrow) and at the posterior side (arrowhead) (C). Expression of IGF-II in the anterior and posterior region of the border between foot and leg of a 11.5 dpc hind limb (arrowheads) and in the marginal vein (arrow) (D). Expression of IGF-II in a 13.5 dpc hind limb in the separate pre-phalanges (arrows) and in the marginal vein (E). Expression of IGF-II in a 14.5 dpc fore limb in the pre-phalangeal cells surrounding the pre phalangeal joint areas (arrows) (F). Expression of IGF-IR in a 11.5 dpc hind limb in the marginal vein (arrow) and in posterior and anterior proximal regions (signal strongest in the anterior region; arrowhead) (G). Signal of Igf1r in a 12.5 dpc fore limb (H). Expression of IGF-IR in a 13.5 dpc hind limb in the pre-cartilage layers located in the pre phalangeal joint areas (arrow) (I).

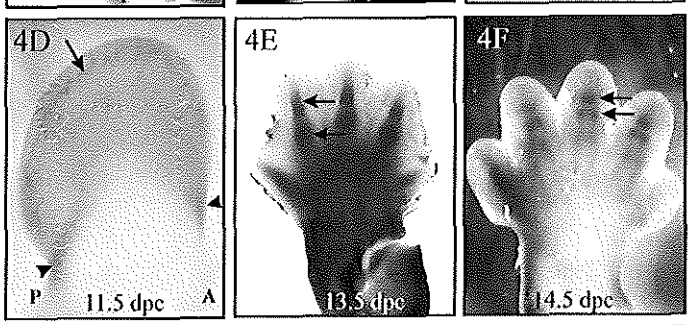
Figure 5
mRNA expression patterns of the IGFBP-2, -3,-4 and -5 in limbs of 9.5-14.5 dpc (A-L). IGFBP-2 in a 9.5 dpc fore limb in the proximal mesoderm (arrow) and in the AER (arrowhead) (A). IGFBP-2 expression in a 13.5 dpc hind limb in the interdigital region (arrows) (B). Expression of IGFBP-2 in a 14.5 dpc hind limb in the borders of the interdigital region (arrowhead) and in the mesodermal cells surrounding the distal part of the pre-phalanges (arrow) (C).
IGFBP-3 expression in a 11.5 dpc fore limb in bloodvessels in the middle of the paddle (arrow) and in the posterior and anterior proximal mesoderm (arrowheads). Note that due to technical limitations the signal has become red (D). The IGFBP-3 expression patterns in a 12.5 dpc fore limb in the proximal interdigital region (arrows) and the borders of the interdigital zones (arrowhead) (E). Expression of IGFBP-3 in a forelimb of 14.5 dpc in the proximal interdigital zone (arrows), in the phalangeal joint areas (asterix) and in the proximal posterior region (arrowhead) (F). IGFBP-4 mRNA in a 10.5 dpc fore limb in the posterior mesoderm (G). IGFBP-4 mRNA localization in a 12.5 dpc fore limb in the proximal interdigital regions (arrows) (H). Sequential expression pattern of IGFBP-4 in a 14.5 dpc hind limb in the pre-phalangeal joint areas (asterixes), proximal posterior expression (arrowhead) and in the proximal interdigital area (arrows) (I). IGFBP-5 expression in the AER of a 11.5 dpc hind limb (J). Sequential expression of IGFBP-5 in a 13.5 dpc hind limb in the proximal interdigital region and in the phalangeal region; halfway the phalangeal region (*), more proximally (**), to disappear finally, except the most proximal signal (***) (K). Expression of IGFBP-5 in the 14.5 dpc hind limb in the proximal part of the interdigital zone (arrows) and in the pre-phalangeal joints (asterixes) (L).



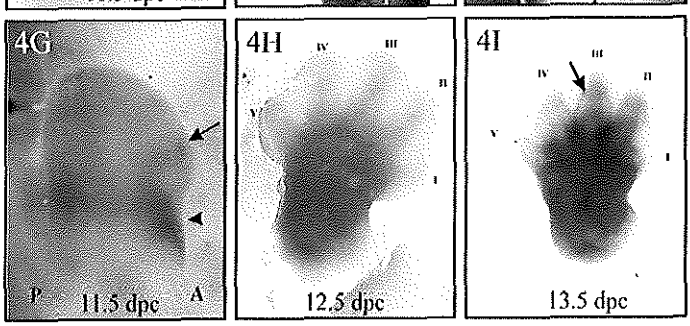
IGF-I

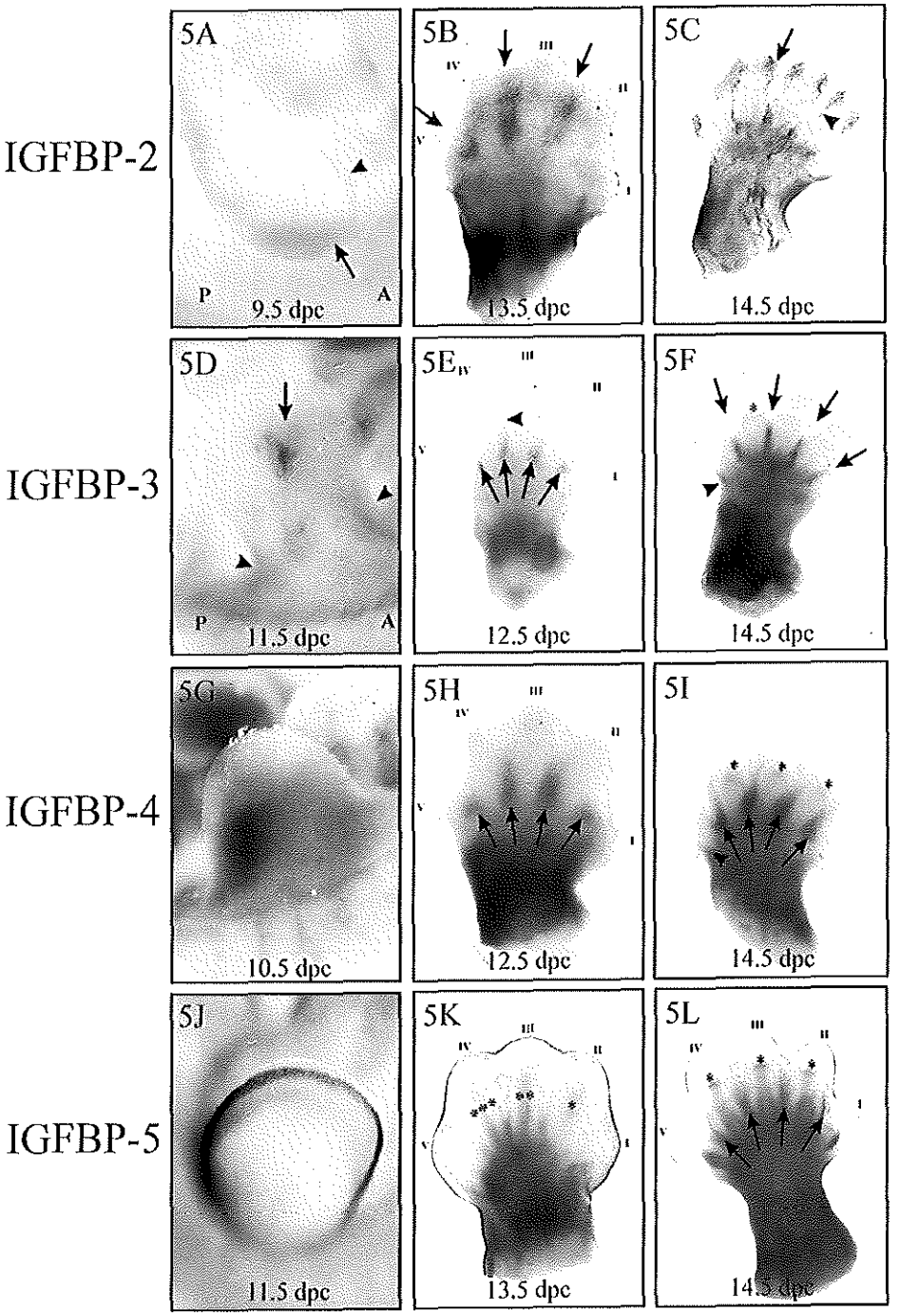


IGF-II



IGF rec.




IGFBP-2
IGFBP-3
IGFBP-4
IGFBP-5
5A
P
9.5 dpc
A
5B
13.5 dpc
5C
14.5 dpc
5D
P
11.5 dpc
A
5E
12.5 dpc
5F
14.5 dpc
5G
10.5 dpc
5H
12.5 dpc
5I
14.5 dpc
5J
11.5 dpc
5K
13.5 dpc
5L
14.5 dpc

Discussion

In this study we investigated the presence of the IGF system during mouse limb development. We analyzed the mRNA expression patterns of IGF-I, IGF-II, IGF-IR, and the six IGFBPs, during different stages of limb development by whole mount *in situ* hybridization.

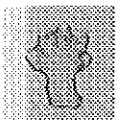
Expression in the AER and ectoderm of the early limb bud

Expression of IGFBP-2 and IGFBP-5 is located in the ectoderm of the limb bud (Streck et al., 1992; our results). Besides IGFBP-2 and IGFBP-5 also fibroblast growth factor-8 (FGF-8) is expressed in the AER, starting at 10 dpc. FGF-8 has been reported to be the first factor being produced in the lateral plate, suggesting an initiating capacity during limb outgrowth of this factor (Crossley et al., 1996). However, in the chick limbless mutant, which lacks FGF-8 expression, a normal AER is formed, indicating the involvement of another factor in limb bud initiation (Ros et al., 1996). Recently, also HOX9 has been shown to be involved in the initiation of limb budding (Cohn et al., 1997). Other genes/gene products involved in early limb development may be retinoic acid (RA) (Helms et al., 1996) or HOXB8 (Charité et al., 1994), which are known to regulate sonic hedgehoc (Shh) expression, or FGF-4, which maintains Shh (Laufer et al. 1994). Shh in turn, is expressed in different organizing centers and is involved in patterning (review Hammerschmid et al., 1997). Furthermore, it has been reported that the effects on outgrowth by the FGFs are dependent on the IGFs (Dealy et al., 1996). However, our study indicates that the IGFs are expressed in the mesoderm, and not in the ridge, where IGFBP-2 and -5 are located from a very early stage (9.5 dpc). This suggests a role for IGFBP-2 and -5 by allocating the IGF peptides from the mesoderm (Ralphs, 1990; our results) into the AER or surface ectoderm.

Expression in the ZPA region

IGF-I and IGFBP-4 are expressed in the posterior mesoderm, or ZPA region. This region is like the AER an important source of signals that controls the antero-posterior patterning of the developing limb (Tickle, 1996). The expression of IGFBP-4 partly resembles the location of Shh and bone morphogenetic protein-2 (Bmp-2) mRNAs (Francis et al., 1994). Shh concentration in the ZPA primarily determines the digit identity, although other signals may also be involved (Hammerschmidt et al., 1997). Bmp-2 is involved in the differentiation and pattern formation in mammals (Lyons et al., 1990) and its expression is activated by Shh (Francis-West et al., 1995). Furthermore, Bmps play a role in the specification of digital and interdigital regions of the limb (Ganan et al., 1996). Moreover, Bmp-2 enhances IGF-I and -II synthesis in skeletal cells (Gabbitas and Canalis, 1995). Thus, there is a possible interaction between IGF-I, IGFBP-4 and the Bmps, specifically in chondrogenic differentiation of mesodermal cells during early limb bud development.

For maintenance of ZPA activity the interaction of FGF-4, Shh and RA is required (Means and Gudas, 1995). Retinoic acid, the active metabolite of vitamin A (retinol), plays a role during mammalian embryogenesis (Wilson et al., 1953) and may have a central role in normal limb morphogenesis (reviewed by Paulsen, 1994). Shh and RA are located in the ZPA, which is also the source of a factor that maintains the thickness and outgrowth-promoting activity of the AER (Saunders & Gasseling, 1968). This may be RA,



that induces factors in the AER, required for the maintenance of Shh and Bmp-2, and for polarizing activity (Helms et al., 1996). In addition, RA induces differentiation and blocks IGF-I mediated growth in various cell types (Li et al., 1994) and has been shown to influence IGFBP levels in many cell systems (Woodward et al., 1996; Zhou et al., 1996). Therefore, these factors may regulate the AER in concert.

Formation of precartilage and joints of the future phalanges

Condensation of the mesoderm, to form the precartilage of the future digits, starts at 12.5-13 dpc (Theiler, 1972). Bmp-2 and -4 are expressed in chondrogenic regions in the later bud, suggesting a role in cartilage differentiation and morphogenesis (Lyons et al., 1989). Also HOXD13 may be involved in growth and patterning of the phalanges (Zakany et al., 1996; Muragaki et al., 1996). We show that both IGF-I and -II, IGF-IR, IGFBP-3, IGFBP-4 and IGFBP-5 mRNA are located in these chondrogenic regions. IGF-II is known to promote chondrocyte, osteoclast, and osteoblast differentiation (Gabbitas et al., 1994). We observed these IGF-II signals were completely covering the precartilage areas in the digit rays at 13.5 and 14.5 dpc.

At 12.5, 13.5 and 14.5 dpc IGF-I is located at the borders of the phalangeal bones. Remarkable is the fact that IGF-I expression first appears between digits III and IV, followed by expression between II and III, IV and V and I and II respectively, following the reported sequence of condensation of the digits (Wanek et al., 1989). IGFBP-2, -3 and -5 show the same sequential expression in the pre-phalanges. However, the localization is slightly different. After 13.5 dpc, the IGF-I signal becomes v-shaped, located at the most proximal border of the interdigital zone. According to the inhibitory role of IGF-I on apoptosis, this may indicate that IGF-I functions as a stop signal for apoptosis of proximal interdigital cells.

IGFBP-4 at 14.5 dpc, is located in the interdigital zone surrounding the proximal precartilage elements. Evidence is emerging that IGF may determine IGFBP-4 availability in bone micro environment (Durham et al., 1995), inhibiting the stimulating effects of IGF-I on bone formation. After formation of the skeletal precursors in the digits, joints will arise by cavity formation by cell death. Little is known about this multi-step process. Growth/differentiation factor-5 (Gdf-5), a BMP-like molecule, is an early marker for cartilage differentiation (Storm et al., 1994) and for joint formation (Storm and Kingsley, 1996). Its expression in joint areas is seen at 13.5 dpc. The sequential expression patterns of IGFBP-3, -4, and -5 in the phalangeal joints regions show remarkable resemblance with the patterns observed when limbs are stained for cell death by Nile blue sulfate (Kimura and Shiota, 1996; our results) or for DNA fragmentation by TUNEL (Zakeri et al., 1994).

The interdigital zone

As apoptosis taking place in the interdigital zone leads to the final shape of the digits, it is of importance to know which molecules are expressed in this region. Interdigital expression has been reported for several genes, including the Tgfβ family members Bmp-2, Bmp-4 (Francis et al., 1994; Zou and Niswander, 1996) and Bmp-7 (Helder et al., 1995), the homeobox containing genes msx-1 and msx-2 (Suzuki et al., 1991; Coelho et al., 1991) and some members of the retinoic acid family (Ruberte et al., 1992). The Bmps have been suggested to act as a signal for apoptosis, in presence of msx and growth factors (Gañan et al., 1996).

Our results show interdigital expression of IGF-I, IGFBP-2, IGFBP-3, IGFBP-4 and IGFBP-5. IGF-I, IGFBP-3 and IGFBP-4 expression in the interdigital zone starts at 12.5 dpc, in the proximal regions, while IGFBP-2 and -5 appear at 13.5 dpc, in more distal parts of the interdigital zone. The IGFBP-2 interdigital pattern corresponds with areas of cell death as is demonstrated by our results of NBS staining.

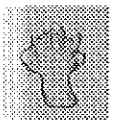
The proximal interdigital expression of IGFBP-4 colocalizes with IGF-I. As of to date IGFBP-4 solely is known to be an inhibitor of IGF-I function, we suggest that its function during the separation of digits may be to counterbalance the inhibitory effects of IGF-I on apoptosis. Furthermore, the interdigital expression patterns for IGFBP-2 at 13.5 dpc overlap with Crbp I (cellular retinol binding protein), a RA-responsive gene (Ruberte et al., 1992).

Finally, the expression of IGF-I, IGFBP-3 and IGFBP-4 in the posterior mesoderm at 13.5-14.5 dpc is difficult to explain. This region, together with the anterior mesoderm and the interdigital mesoderm, has the potential to form extra digits, prior to the occuring cell death (Hurle et al. 1989). IGF-I, IGFBP-3 and IGFBP-4 are indeed expressed in the (proximal) interdigital mesoderm, where they colocalize with eachother. However, no anterior expression is found for these genes.

It has been suggested that the survival of the interdigital mesenchyme is dependent on the continuous influence of a trophic factor, probably located in the ectoderm, which sustains its proliferation (Macias et al., 1996). IGFBP-5 is expressed strongly in the ectoderm of the limb bud during all stages studied in this experiment. At 13.5 and 14.5 dpc, when apoptosis peaks in the interdigital zone (Zakeri et al., 1993), IGFBP-5 expression in the interdigital area may serve to regulate IGF-I inhibition of apoptosis. We suggest that IGFBP-5 is a survival factor for both the interdigital mesenchyme and the ectoderm.

In conclusion, IGF system members show differential, temporal and spatial expression patterns, pointing to different functions during limb development and colocalize with important signalling regions during early limb development. We also show (partly) colocalization of members of the IGF system with NBS staining, indicating regions with cell death. Finally, localization of the IGF system is found in regions, in which mesodermal condensation takes place, to form precartilage.

Therefore, we hypothesize that the members of the IGF system may be involved in chondrogenic differentiation of mesoderm and the regulation of apoptosis in the developing limb. Not much is known about the involvement of the IGF system in the early differentiation of bone. The fact that some of the members of the IGF system are localized in regions known to express molecules that determine pattern formation in the developing limb and colocalize with factors which act as morphogens in early bone formation, supports our hypothesis. Moreover, the amount of growth factors locally available has been reported to influence the induction of apoptosis (review White 1996 and references therein). IGFBPs are able to influence the amount of effective IGF available, and may thereby form part of the mechanism that controls apoptosis. However, not much is known about the possible relationships between the IGF-system and other factors and it will be necessary to concentrate on elucidating these interactions during limb development.



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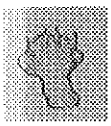

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Generation of polyclonal antibodies to mouse insulin-like growth factor binding proteins (IGFBP)-1 to -6 and detection of IGFBP protein and mRNA in the mouse embryo

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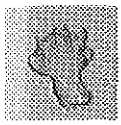


Abstract

The IGF system is an important regulator of embryonic/fetal growth and differentiation. IGF bioavailability is modulated by IGF binding proteins (IGFBPs). We have generated six different polyclonal antibodies, directed against mIGFBP-1 through -6. Specific antibodies were generated, directed to synthetic peptide fragments of the IGFBPs, that detect mouse IGFBP protein tissue localization. The specificity of the produced polyclonals was demonstrated by ELISA, Western blotting and immunohistochemistry. In addition, correlation of protein and mRNA patterns were analyzed.

Immunohistochemistry and mRNA *in situ* hybridization of sections of mouse embryos of 13.5 days post coitum (dpc) revealed tissue-specific expression patterns for the six IGFBPs. The only site of IGFBP-1 protein and mRNA production was the liver. IGFBP-2, -4 and -5 protein and mRNA were detected in various organs and tissues. When expressed, in various tissues, IGFBP-3 and -6 protein and mRNA levels were low. In several tissues, such as lung, liver, kidney and tongue, more than one IGFBP (protein and mRNA) could be detected. Differences between mRNA and protein localization were found for IGFBP-3, -5 and -6, suggesting that these IGFBPs are secreted and transported.

These results confirm the different spatial localization of the IGFBPs, on the mRNA and protein levels. The overlapping mRNA and protein localization, on the other hand, indicate that the IGFBPs may act as auto- or paracrine regulators of IGF action during mouse development.

Introduction

The IGFBPs are a family of at least six highly homologous proteins that bind IGF with high affinity (Jones and Clemmons 1995). Together with the IGF receptors, IGFs and IGFBPs form the IGF system that is important during development, where IGFBPs serve as regulators of IGF bioavailability (Ludwig et al., 1996; Liu et al., 1993a/b; De Chiara et al., 1990). Human fetal tissues synthesize and secrete IGFBPs in a tissue-specific fashion (Pannier et al. 1994; Hill & Clemmons 1992, Hill et al 1989, Han et al. 1987).

Mouse models are widely used to obtain insight into the actions of the IGF system members during development (van Kleffens et al., 1998b; Adesanya et al., 1998; Erickson et al., 1998; Hill et al., 1998; Pintar et al., 1998). Gene expression studies have shown that the IGF system components have specific spatial and temporal mRNA expression patterns during development (van Kleffens et al., 1998a; Lindenbergh-Kortleve et al., 1997; Schuller et al., 1994; Green et al., 1994; Cerro et al., 1993; Wood et al., 1992). As the IGFs and IGFBPs are secreted proteins, it is relevant to extend analysis of gene transcription to the localization of the proteins at their site of action.

Several studies have confirmed the tissue specific protein localization of the components of the IGF system in human (Hill et al., 1992; Hill et al., 1989; Han et al., 1987). The existing antibodies to human and rat IGFBPs, however, are not applicable in immunohistochemical detection in mice (Liu et al., 1993c).

Therefore, the aim of this study was to generate specific polyclonal antibodies against the mIGFBPs and to compare IGFBP protein localization patterns with IGFBP mRNA patterns during mouse development. The specific mIGFBP antibodies were raised using synthetic peptides specific for each of the IGFBPs and were characterized by ELISA, Western blotting and immunohistochemistry. Immunohistochemistry and *in situ* hybridization were performed on sections of 13.5 dpc mouse embryos to analyze protein and mRNA localization of the six IGFBPs.

Materials and Methods

Generation of polyclonal antibodies

Polyclonal antibodies to mIGFBP-1, -2, -3, -4, -5 and -6 were generated using synthetic peptides (ID-DLO, Lelystad, The Netherlands) (Table 1). These peptides were chosen from the unique middle part of the IGFBP amino acid sequences, to limit cross reactivity between the various IGFBPs. The choice of the peptide fragments was guided by hydrophobicity plots (Kyte and Doolittle, 1982), surface probability predictions according to Emini, Chou-Fasman secundary structure predictions (Fasman and Chou, 1976) and antigenicity index (Jameson and Wolf, 1988). A terminal cystein was added to the peptide for sulfhydryl coupling. Conjugation to the carrier keyhole limpet hemocyanin (KLH) was performed following the manufacturer's prescription (Pierce, Rockford, Illinois USA). KLH coupled peptides were injected subcutaneously into rabbits (0.25 mg), using specol (Central Veterinary Institute, Lelystad, The Netherlands) as adjuvans. Three weeks after primary immunization the rabbits were boosted (protocol similar to primary immunization). After a final boost, three weeks later (similar to previous boost), sera were collected and used in subsequent experiments.

Table 1

Animo acid sequences of the synthetic peptide fragments of the IGFBPs used to produce anti-mIGFBP antibodies

IGFBP-1	MRAREIADLKKWKEPC
IGFBP-2	CEKVNEQHRQMGKGAKH
IGFBP-3	FSSESKRETEYGPC
IGFBP-4	VGTPREEPRPVPQGSC
IGFBP-5	VIPAPEMRQESEQGPC
IGFBP-6	CSRDTNHRDRQKNPRTS

ELISA

96 Microwell plates were coated with peptide (without KLH), 2mg/ml in coupling buffer (50 mM carbonate buffer pH 9.0) and dried overnight at 37°C. After blocking with 3% BSA (fraction V; Boehringer Mannheim, GmbH, Mannheim, Germany) in PBS dilution gradients of the antisera were pipetted into the wells (1:500 to 1:16,000), which were incubated overnight at 4°C. A peroxidase conjugated swine anti-rabbit antibody (DAKO, Glostrup, Denmark) was used as a secondary step (1:1,000) during 1 hour. Detection was done with o-phenyl diamine (OPD; Eastman Kodak, Rochester, NY, USA) (20mg OPD + 50ml H_2O_2/10 ml 0.1M citric acid, 0.2M Na_2HPO_4, pH5.0). The reaction was stopped with 5M H_2SO_4.

Negative controls were included, consisting of incubation of the wells plate, coated with peptides, with pre-immune sera of the respective antisera, in the same dilutions. Cross-reactivity was checked by incubating each peptide with all IGFBP antibodies (1:500 and 1:1,000).

Western blotting

Murine erythroleukaemia (MEL) cells were transfected with IGFBP-1, 2, 4 and 6 cDNAs (Schuller et al., 1994), according to Needham and coworkers (1992). In the log phase these cells were induced for 48h with DMSO. Subsequently, cell culture medium was TCA precipitated (50 v/v 20%trichloric acid, overnight at 4°C) (Merck, Darmstadt, F.R. Germany). IGFBP production was tested by IGF-ligand blotting as described by Hossenlopp et al. (1985).

For IGFBP-3 normal mouse serum was used, whereas for IGFBP-5 total protein was extracted from fetal mouse lungs (13.5 dpc) by the TRIzol method, following the manufacturer's prescription (Life Technologies, Inc., Rockville, MD, USA).

Proteins were separated on a reducing 8% or 10% SDS:PAGE and transferred either to pvdf (Millipore, Bedford, USA) or nitrocellulose (Schleicher & Schuell, Dassel, Germany) membranes. The pvdf membranes were blocked with 5% non-fat dry milk (Profilar, Nutricia, Zoetermeer, The Netherlands), the nitrocellulose membranes with 3%BSA (Boehringer Mannheim, GmbH, Mannheim, Germany). Membranes were incubated overnight at 4°C with the primary antibody (dilutions varying from 1:200 to 1:20,000). As a secondary step a peroxidase conjugated swine anti-rabbit antibody was used (DAKO, A/S, Glostrup, Denmark) 1:2,000) on the pvdf membranes, 1 hour at room temperature. The nitrocellulose membranes were incubated with a secondary alkalic phosphatase antibody (DAKO, A/S, Glostrup, Denmark). Pvdf membranes were incubated according to the manufacturer's procedure (Pierce, Rockford, Illinois USA) and subsequently covered with ECL hyperfilms (Amersham Lifesciense, Buckinghamshire, England). Films were illuminated varying from 1 minute to 2 hours, depending on the intensity of the signal. Detection on nitrocellulose membranes took place with NBT (4-nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate).

Immunohistochemistry on sections

Balb/c mice were mated and the morning of appearance of a vaginal plug was assigned 0.5 days post coitum (dpc). Pregnant females were killed by cervical dislocation and embryos were collected at 13.5 dpc and fixed overnight in 4% paraformaldehyde. After embedding of the fixed embryos in paraffin, 5 µm sections were cut and mounted onto aminopropyl trioxysilane (AAS) coated slides. Immunohistochemistry was performed making use of a peroxidase-anti-peroxidase (PAP) method or an avidine-biotine complex (ABC) method. The anti mIGFBP-1 and -3 polyclonal antibody sera were diluted 1:150, while the anti mIGFBP-2, -4, -5 and -6 polyclonal antibodies were used in a dilution of 1:250. Unlabeled goat anti-rabbit immunoglobins (1:50) (DAKO, Glostrup, Denmark) were used as second antibody and rabbit PAP (1:100) (DAKO, Glostrup, Denmark) as a linker in the PAP method. Incubation with a biotine conjugated goat anti-rabbit (DAKO, Glostrup, Denmark) completed with a streptavidin horseradish peroxidase complex (Biogenex, San Ramon, CA, USA) incubation was used for the ABC method. Staining was performed with diaminobenzidine (0.75 mg/ml) (DAB, Fluka Chemika, Buchs, Switzerland). Nuclei were visualized with Mayer's haematoxilin. The tissues were analyzed under light microscopy. Controls were performed on sections with pre-immune sera of each rabbit, diluted 1:10 or 1:50. As a second control the immune sera were mixed in a dilution range with the corresponding peptides (1, 0.5 and 0.17µg/µl) and incubated overnight at 4°C. With these mixtures immunohistochemistry on sections of mouse embryos was performed.

Probe preparation (in situ hybridization)

Specific mouse IGFBP cRNA probes were transcribed from IGFBP-2, -3, -4, -5, -6 as described by Schuller et al. (1994). The mouse cDNA *SphI-SacI* fragment was used as template for the IGFBP-1 cRNA probe and was cloned into pTZ18R or pTZ19R (Pharmacia, Uppsala, Sweden) for the antisense and sense probes, respectively. Digoxigenin-11-UTP labeled RNA probes were prepared according to the manufacturer's prescription (Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany), using T7 or SP6 RNA polymerase.

In situ hybridization

A non-radioactive *in situ* hybridization was performed, as described before (Lindenbergh-Kortleve et al., 1997). Hybridization was performed overnight at 55 °C in a humid chamber in a hybridization solution containing 50% deionized formamide, 10% dextran sulphate, 2xSSC, 1xDenhardt's solution, 1µg/ml tRNA, 250 µg/ml herring sperm DNA and the respective probes at a concentration of 100 ng/ml. After various washes (50% formamide/2xSSC, 50% formamide/1xSSC and 0.1xSSC) sections were incubated with RNase T1 (2U/ml in 1mM EDTA/2xSSC) for 15 minutes at 37°C. The digoxigenin labeled hybrids were detected by antibody incubation performed according to the manufacturer's recommendations (Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany). PVA (Polyvinylalcohol, MW 31-50 kD, Aldrich Chemical Co. Milwaukee, WIS, USA) enhanced staining with BCIP (5-bromo-4-chloro-3-indolyl-phosphate), NBT (4-nitroblue tetrazolium chloride) and levamisol was stopped when the desired intensity of the blue precipitate was reached. This was dependent on the relative abundancy of the respective mRNAs. Sections were counterstained with Nuclear red solution and mounted with Euparal (ChromaGesellschaft, Stuttgart, Germany). Negative controls for *in situ* hybridization were performed using sense probes, which never gave any significant staining.

Results

Analysis of antibody specificity and cross-reactivity

After immunization and boost all rabbits developed antisera against the predicted mIGFBP. As is shown in Figure 1, ELISA assay of the antisera demonstrated a specific response to the injected peptides. Antisera against mIGFBP-1, -3, -4 and -6 were very specific and already demonstrated a clear color reaction at a dilution to 1:8,000. Clear color reactions of antisera against IGFBP-2 and -5 were obtained at a dilution of 1:4,000. As a control, incubation with each of the pre-immune sera in a dilution range was included that showed no significant staining. No cross-reactivity between the antisera and the IGFBP peptide fragments was observed (data not shown).

Detection of mIGFBPs on Western blot was possible for mIGFBP-1, -3 and -5 (Fig. 2). The patterns on Western blot corresponded with the IGF-ligandblot results (data not shown). A distinct band of approximately 30 kDa was visible for mIGFBP-1, after dilution of the antibody to 1:20,000. When 1µl mouse serum was loaded the IGFBP-3 43-45 kDa doublet was visible after incubation with the anti-IGFBP-3 antibody (diluted 1:10,000). Loading of total protein of 13.5 dpc mouse lungs gave a band of approximately 29 kDa when incubated with the IGFBP-5 antibody (1:1,000). Neither of the antibodies showed cross-reactivity.

Specificity of immunohistochemical detection of IGFBPs in mouse embryonal tissue

Specificity of the generated mIGFBP antisera in immunohistochemistry was checked by incubating the antisera overnight with the corresponding synthetic peptides in a dilution range (60-10ng/ml). Subsequently, these mixtures were used in an immunohistochemical procedure on sections of 13.5 dpc mouse embryos. This resulted in a gradual loss of signal with increasing peptide concentration. An example is given in figure 3. This loss of signal did not occur when a random peptide was used (data not shown).

Comparison of protein and mRNA localization of the six IGFBPs in the 13.5 dpc mouse embryo

The antisera against mIGFBP-1 through -6 were applied to paraffin sections of mouse embryos (13.5 dpc) to localize mIGFBP proteins. Similarly, mRNA expression patterns in 13.5 dpc mouse embryos were determined by *in situ* hybridization. *In situ* hybridization and immunohistochemistry staining patterns are summarized in Table 2.

IGFBP-1 gene expression was limited. Staining of IGFBP-1 protein was visualized only in the liver, similar to IGFBP-1 mRNA expression (Fig. 3b, 4a).

Like IGFBP-2 mRNA expression, IGFBP-2 protein occurrence was diverse. Major sites of IGFBP-2 protein localization were liver, kidney, choroid plexus, lung and floor plate. This is in accordance with mRNA expression. Both IGFBP-2 mRNA and protein were located in the epithelial cells of the kidney and choroid plexus (Fig. 4b-d). In lung, IGFBP-2 mRNA and protein were present in the bronchi, but also scattered in underlying mesenchymal cells (Fig. 4e,f). However, there was a discrepancy between the bronchial localization of IGFBP-2 mRNA and protein. The mRNA was located in the epithelial cells, while IGFBP-2 protein was detected in the underlying bronchial cells (Fig. 4e,f).

IGFBP-3 mRNA expression was weak and colocalized with IGFBP-3 protein in liver, tongue, lung and in nose epithelium (very weak signal) (Fig. 4g-j). In the tongue IGFBP-3 mRNA and protein were not colocalizing, but expressed in neighbouring cell types in muscle (Fig. 4i,j). In contrast to mRNA expression, IGFBP-3 protein also could be detected in the choroid plexus and kidney, whereas mRNA expression could also be detected in the heart.

In accordance with mRNA expression, staining of IGFBP-4 protein was detected widespread. Overlapping mRNA and protein expression was visualized in mesencephalon (Fig. 4k,l), telencephalon, heart, liver (Fig. 4m,n), lung, tongue mesenchyme and bloodvessels. In addition to the mRNA expression profiles, IGFBP-4 protein could be demonstrated in the kidney.

IGFBP-5 mRNA was expressed abundantly and was largely overlapping protein occurrence. Identical to mRNA expression IGFBP-5 protein was localized in the liver, mesenchym underneath the epithelium of the gut, meninges, bronchial epithelium and mesenchyme of the lung (very weak protein staining) (Fig. 4o,p) and tongue. In addition, protein was detected in the notochord, the floor plate and muscle.

Colocalization of IGFBP-6 mRNA and protein was observed in nasal epithelium and liver (Fig. 4q,r). Protein was not detected in the perichondral layer of cartilage, as was found for mRNA. In addition, IGFBP-6 protein was visualized in hindbrain and the heart.

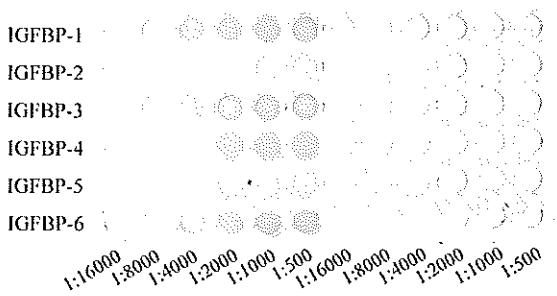
Figure 1
ELISA in which synthetic peptides are coated to the wells plate and corresponding immune and pre-immune sera are tested in a dilution gradient (see materials and methods). Each horizontal row represents a different IGFBP antiserum (immune and pre-immune). Vertical rows are divided into immune and pre-immune sera, which are subdivided into various dilutions. The left and right side of the ELISA plate are incubated with immune and pre-immune sera, respectively. Sera are diluted from 1:500 to 1:16,000.

Figure 2
Western immunoblots of mIGFBP-1, -3 and -5. mIGFBP-1: 100µg IGFBP-1 from MEL cell medium (ECL). Dilution of the mIGFBP-1 antibody: 1:20,000. IGFBP-3: 1µl normal mouse serum (ECL). Dilution of the mIGFBP-3 antibody: 1:10,000. IGFBP-5: total protein (80µg) of 13.5 dpc mouse lungs (NBT/BCIP staining). Dilution of the mIGFBP-5 antibody: 1:1,000.

Figure 3
Immunohistochemistry on 13.5 dpc mouse embryo, after overnight blocking of the IGFBP antisera with different dilutions of the corresponding synthetic peptide. a. IGFBP-1 protein in liver. Antiserum against IGFBP-1 (1:250) was blocked with 60 ng corresponding peptide per slide. b. IGFBP-1 protein in liver. Antiserum against IGFBP-1: 1:250. Arrows: weak immunohistochemical staining of IGFBP-1 in hepatocytes.

Figure 4
Immunohistochemical detection of IGFBP mRNA (blue) and proteins (brown) in the mouse embryo (13.5 dpc). 4a: IGFBP-1 mRNA in liver. 4b: IGFBP-2 protein in the choroid plexus. Arrows: IGFBP-2 protein in epithelial cells. 4c: choroid plexus, pre-immune serum as a negative control for IGFBP-2 (1:10). 4d: IGFBP-2 mRNA in the choroid plexus. 4e: IGFBP-2 protein in lung. Arrow: IGFBP-2 protein in bronchial cells. Arrowhead: IGFBP-2 protein in mesenchymal cells. 4f: IGFBP-2 mRNA in lung. Arrow: localization in epithelial cells. Arrowhead: localization in mesenchymal cells. 4g: IGFBP-3 protein in liver. 4h: IGFBP-3 mRNA in liver. 4i: IGFBP-3 protein in tongue (muscle). 4j: IGFBP-3 mRNA neighbour IGFBP-3 protein in tongue. 4k: IGFBP-4 protein in mesencephalon. 4l: IGFBP-4 mRNA in mesencephalon. 4m: IGFBP-4 protein in liver. 4n: IGFBP-4 mRNA expression in the liver. 4o: IGFBP-5 protein in lung. 4p: IGFBP-5 mRNA in lung. 4q: IGFBP-6 protein in nasal epithelium. 4r: IGFBP-6 mRNA in liver.

Fig. 1


Immune sera
Pre-immune sera
IGFBP-1
IGFBP-2
IGFBP-3
IGFBP-4
IGFBP-5
IGFBP-6
1:16000
1:8000
1:4000
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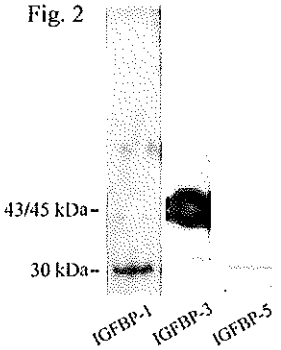
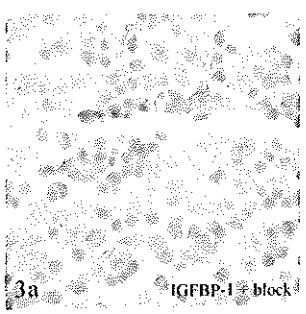
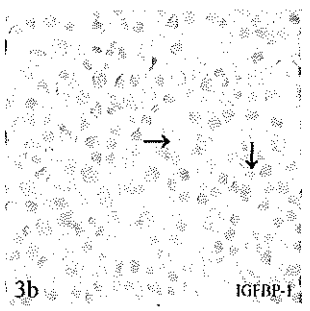
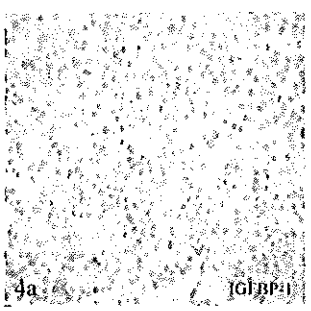
1:1000
1:500
1:16000
1:8000
1:4000
1:2000
1:1000
1:500

Fig. 2


43/45 kDa-
30 kDa-
IGFBP-1
IGFBP-3
IGFBP-5


3a
IGFBP-1 block


3b
IGFBP-1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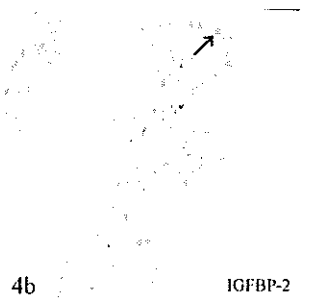
IGFBP-1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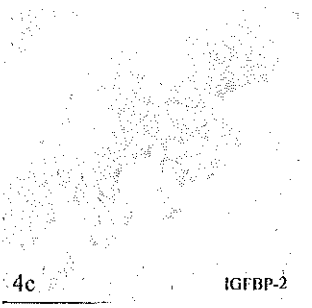
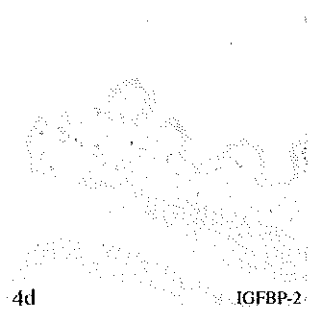
IGFBP-2


4c
IGFBP-2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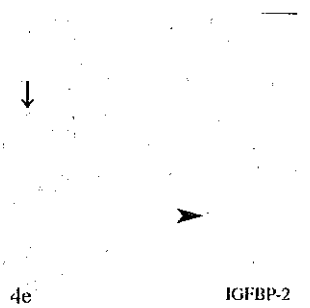
IGFBP-2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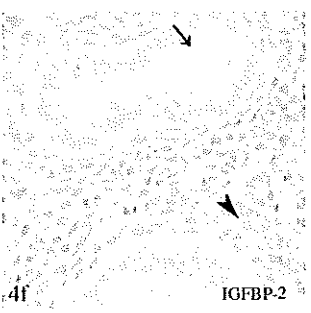
IGFBP-2


4f
IGFBP-2

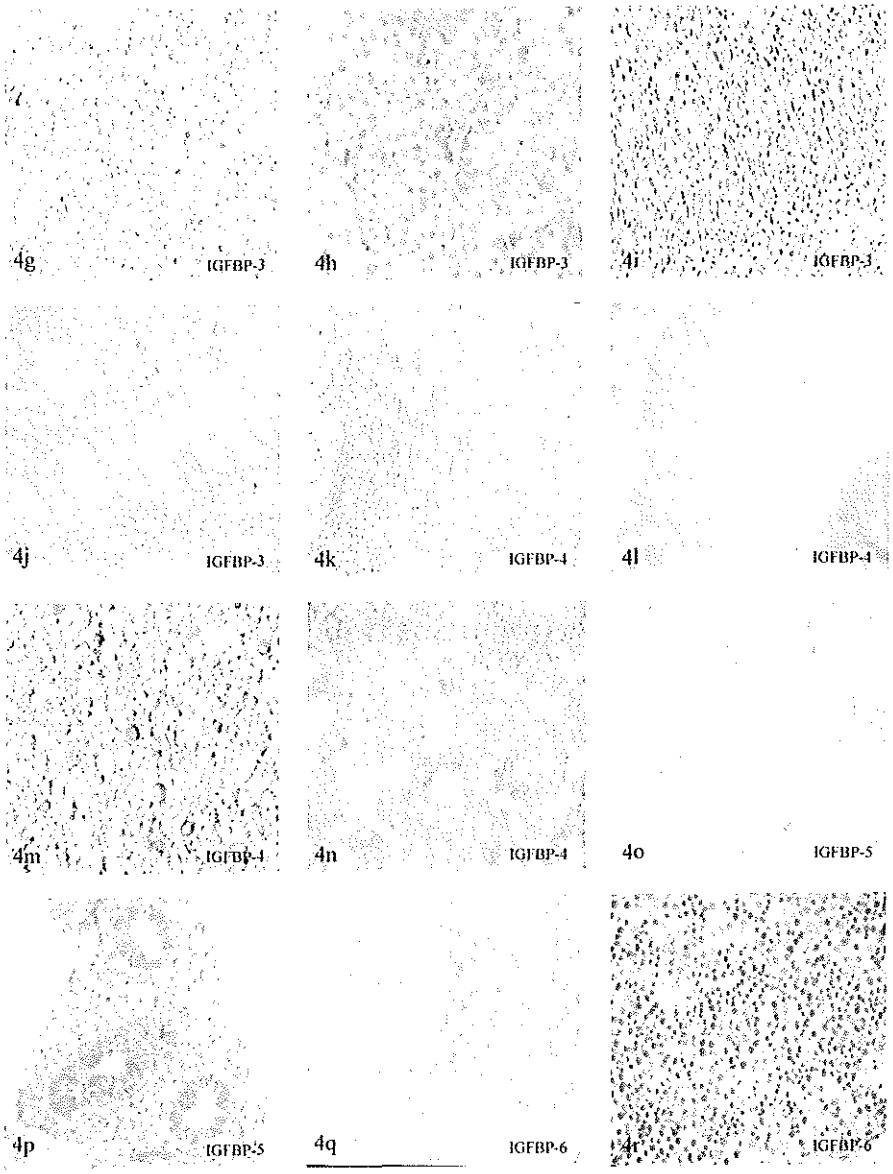

4g IGFBP-3
4h IGFBP-3
4i IGFBP-3
4j IGFBP-3
4k IGFBP-4
4l IGFBP-4
4m IGFBP-4
4n IGFBP-4
4o IGFBP-5
4p IGFBP-5
4q IGFBP-6
4r IGFBP-6

Table 2

Localization of protein and mRNA of IGFBP-1 through -6 in different tissues and organs of the 13.5 dpc mouse embryo

	protein	mRNA
IGFBP-1	liver	liver
IGFBP-2	lung	lung
	liver	liver
	kidney	kidney
	choroid plexus	choroid plexus
	floor plate	floor plate
IGFBP-3	liver	liver
	tongue	tongue
	lung	lung
	nasal epithelium	nasal epithelium
		heart
	choroid plexus	
	kidney	
IGFBP-4	mesencephalon	mesencephalon
	telencephalon	telencephalon
	heart	heart
	liver	liver
	lung	lung
	tongue	tongue
	bloodvessels	bloodvessels
	kidney	
IGFBP-5	liver	liver
	gut endothelium	gut endothelium
	meninges	meninges
	lung mesemchyme	lung mesenchyme
	tongue	tongue
	notochord	
	floor plate	
	muscle	
IGFBP-6	liver	liver
	nasal epithelium	nasal epithelium
		perichondral layers of cartilage
	hindbrain	
	heart	

Discussion

To analyze IGFBP protein localization in mouse embryonal tissues we generated specific polyclonal antibodies against mIGFBP-1 through -6. The specificity of these antibodies was demonstrated by ELISA, immunohistochemistry and Western blotting (for IGFBP-1, -3 and -5). The fact that the antibodies against mIGFBP-2, -4 and -6 were not able to detect the corresponding IGFBP on Western blot is probably due to conformational changes of the protein induced by the Western-blot procedure.

With the six antibodies immunohistochemical localization of mouse IGFBP proteins (mIGFBP-1 to -6) was compared with the mRNA patterns obtained with *in situ* hybridization of sections of the mid gestational mouse embryo. These data extend our former gene expression studies, that describe the tissue-specific mRNA expression of IGFBP-1 through -6 in the mouse embryo (van Kleffens et al., 1998a; Lindenbergh-Kortleve et al., 1997; Schuller et al., 1995, 1994, 1993).

Our results show that, similar to mRNA expression patterns, localization of IGFBP proteins in the mouse mid gestational embryo is tissue-specific. IGFBP-1 mRNA and protein were detected primarily in the liver, while IGFBP-2, -4 and -5 were distributed among various tissues. Conform the low IGFBP-1, -3 and -6 mRNA levels, these IGFBPs were more difficult to detect than the other IGFBPs.

The limited data available on IGF system mRNA and protein expression during human development are in confirmance with our data (Han et al., 1996; Hill and Clemmons, 1992; Hill et al., 1989; Han et al., 1987).

Although protein localization of the IGFBPs appeared to be mostly similar to mRNA expression, some differences in localization in the 13.5 dpc mouse embryo were demonstrated.

Differences between IGFBP mRNA and protein localization may reveal detection thresholds for either mRNA or protein, or secretion of the protein without binding to the target organ (and protein is then probably washed out during the immunohistochemical procedure). Discrepancy between protein and mRNA expression was seen within tissues. In lung, IGFBP-2 mRNA was detected in bronchial epithelium. However, IGFBP-2 protein was localized in the surrounding bronchial cells. Similar, IGFBP-3 mRNA and protein were located in neighbouring cells in muscles of the tongue. Apparently, in these cases mRNA expression in a given cell type gives rise to protein localization in a cell type that is in close contact. This may point to a mechanism of action where a certain cell type makes and secretes an IGFBP that acts at another (neighbouring) cell type.

Furthermore, substantial differences between mRNA and protein localization were found for IGFBP-3, -5 and -6. IGFBP-3 is the major circulatory IGFBP (Jones and Clemmons 1995) and after its secretion in liver and the cardiovasculair system IGFBP-3 can easily move throughout the embryo/fetus. The IGFBPs may be transported to specific sites, but the differences also may indicate that the mRNA is very labile in these specific tissues, and hence present in concentrations below detection limits. In that case, translation should be efficient and the protein must be very stable to enable protein detection.

Whereas mRNA and protein expression of the different IGFBPs was clearly distinct, most tissues were found to express more than one IGFBP. Examples are liver, lung, heart and tongue. Two possible explanations for this phenomenom exist: all IGFBPs have a specific function and each contribution is necessary for a funtional organ or all IGFBPs have similar funtions and show redundancy.

The fact that IGFBP knock-outs do not show dramatic phenotypes (Pintar et al. 1998; Pintar et al 1995) may suggest overlapping function or redundancy. The IGFBP-2 knock-outs are characterized by an enlarged spleen and changed IGFBP serum levels, probably to compensate for the lack of IGFBP-2 (Pintar et al. 1995). The body weight and body length of the IGFBP-4 knock-outs are slightly smaller than of wild-type mice (Pintar et al 1998). However, these minor changes seen can not be explained by changed patterns of IGFBP expression in tissues of interest.

The tissue-specificity of the IGFBPs and the consistence between IGFBP mRNA and protein localization patterns strongly suggest the IGFBPs (except IGFBP-3) functioning as autocrine or paracrine regulators of IGF actions. The fact that several tissues express more than one IGFBP can also be explained as an indication for the complex regulation of the IGF system.

Summarizing, the generated antisera against mIGFBP-1 through -6 demonstrated specificity in ELISA, Western-blot and immunohistochemistry. With these antisera IGFBP protein localization could be compared to IGFBP mRNA expression patterns in 13.5 dpc mouse embryos. This revealed tissue-specific and consistent mRNA and protein localization, in confirmation with the suggested para- and autocrine functions of the IGFBPs on IGF action.

We anticipate that this set of specific polyclonal antibodies may become an important tool for future IGFBP studies in the mouse.

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Chapter 4

mRNA and protein localization of the IGF system during mouse embryonic development in areas with apoptosis

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Submitted

Abstract

We analyzed mRNA and protein localization of the different components of the IGF system in regions with apoptotis, during mouse development between 9.5 and 13.5 days post coitum (dpc). A spatio-temporal relationship between these expression patterns and the onset of apoptosis in specific areas was sought. The IGFBP mRNA and protein expression patterns were tissue-specific. In most tissues, mRNA expression patterns correlated with protein localization. Discrepancies between mRNA and protein detection were found in e.g. lens, neural layer of the retina, whiskers and somites. Localization of the IGFs, the type I IGF receptor and IGFBP-2 correlated well with cell death regions. When these genes were expressed, no apoptosis occurred, and *vice versa*. Correlation of IGFBP-3, -4 and -5 with apoptosis regions was noticed only at 13.5 dpc. In eye muscles, whiskers and somites, the expression of IGF system components preceded the occurrence of Tunel stained apoptotic cells. When IGF-I expression ceased, apoptosis occurred in these areas. In conclusion, our results suggest that IGF-I, the type I IGF receptor, and IGFBP-2 inhibit apoptosis. In contrast, IGFBP-3, -4 and -5 may stimulate apoptosis by trapping the IGFs. Tissue-specific modulation of IGF protective action against apoptosis by the different IGFBPs during mouse embryonal development may contribute to development of organ specific morphology.

Introduction

Insulin-like growth factors (IGF-I and -II) are single chain polypeptides involved in cell proliferation and differentiation (Jones and Clemmons 1995). Their mitogenic effects are mediated primarily by the type I IGF receptor. In extracellular fluids, IGFs are complexed to IGF-binding proteins (IGFBPs), that are thought to modulate IGF action. Six IGFBPs have been characterized (IGFBP-1 to-6) and more recently several other IGFBP related proteins, with reduced affinity for IGF, have been described (Oh et al., 1996; Collet et al., 1998).

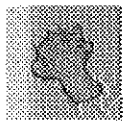
During embryogenesis the IGFs, type I IGF receptor and IGFBP-1 to -6 show unique expression patterns (Schuller et al., 1993; Cerro et al., 1993; Lindenbergh-Kortleve et al., 1997; van Kleffens et al., 1998), indicating that the IGF system participates in developmental processes. Developmental processes are characterized by cell proliferation and differentiation and also apoptosis, i.e. programmed cell death, is an important characteristic of tissue remodeling during ontogeny. It has been shown that IGF system components either inhibit (IGF-I and type I IGF receptor) or stimulate (IGFBP-3 and -5) apoptosis in several *in vitro* and *in vivo* systems (Resnicoff et al., 1995a/b; Chun et al., 1994; Phillips et al., 1994; Guenette and Tenniswood, 1995; Tonner et al., 1995). Recently, we showed distinct spatial and temporal expression patterns for most members of the IGF system during limb development (van Kleffens et al., 1998). For example, we described the specific expression of IGFBP-2 and -5 in the interdigital zone, a site where apoptosis is responsible for the final shape of the digits.

The aim of this study was to find a correlation between the expression patterns of specific members of the IGF system and the onset of apoptosis at specific places during mouse embryogenesis. Therefore, we investigated the expression patterns of the IGF system during mouse development in key areas of apoptosis, for example in the head-neck area and the limbs. Furthermore, we correlated mRNA patterns with protein localization, as the IGFs and the IGFBPs are secreted proteins. *In situ* hybridization, immunohistochemistry and Tunel analysis on consecutive sections allowed us to compare the expression patterns of the IGF system genes, their proteins and apoptosis patterns. In addition, whole mount *in situ* hybridization and Nile Blue staining of whole embryos were used to confirm the data provided by the analysis of sections.

Materials and Methods

Animals and experimental design

Balb/c mice were mated and the morning of appearance of a vaginal plug was assigned as 0.5 days post coitum (dpc). Pregnant females were killed by cervical dislocation and embryos were collected at 9.5 (n=44), 11.5 (n=49), 12.5 (n=5) and 13.5 dpc (n=49) and either directly stained with Nile Blue sulfate (11.5 dpc, n=5; 12.5 dpc n=5; 13.5 dpc, n=5) or fixed overnight in 4% paraformaldehyde. After embedding in paraffin, the fixed embryos were serially sectioned. The 5 µm thick sections were mounted onto aminopropyl trioxysilane (AAS) coated slides for *in situ* hybridization (ISH), immunohistochemistry and Tunel (n=9 per timepoint). Successive sagittal sections were separately mounted onto one of four separate AAS coated slides. Therefore, each slide consisted of complete series of every fourth consecutive section. The non-embedded paraformaldehyde fixed embryos (n=35) were used for whole mount *in situ* hybridization.



Preparation of RNA probes

Sense and anti-sense RNA digoxygenin probes for IGFBP-1 to -6, IGF-I, IGF-II and Type I IGF receptor were generated following the manufacturers procedures (Boehringer Mannheim GmbH, Mannheim, Germany). The cDNAs of the IGF system genes were obtained as described earlier (van Kleffens et al., 1998). Testing of the transcripts was done by agarose gel electrophoresis, blotting, incubation with an alkaline phosphatase conjugated anti-DIG antibody (Boehringer Mannheim GmbH, Mannheim, Germany) and staining with NBT (4-nitroblue tetrazolium chloride; Boehringer Mannheim GmbH, Mannheim Germany) and BCIP (5-bromo-4chloro-3-indolyl-phosphate; Boehringer Mannheim GmbH, Mannheim, Germany).

In situ hybridization

In situ hybridization was performed according to Lindenbergh-Kortleve et al., 1997. Briefly, sections were hybridized overnight at 55°C in a humid chamber in a solution containing 50% deionized formamide, 10% dextran sulphate, 2xSSC, 1xDenhardt's solution, 1µg/ml tRNA, 250 µg/ml herring sperm DNA and the respective probes at a concentration of 100 ng/ml. After various washes (50% formamide/2xSSC, 50% formamide/1xSSC and 0.1xSSC) sections were incubated with RNase T1 (2U/ml in 1mM EDTA/2xSSC) for 15 minutes at 37°C. The digoxigenin labeled hybrids were detected by antibody incubation performed according to the manufacturer's prescription (Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany). PVA- (Polyvinylalcohol, MW 31-50 kD, Aldrich Chemical Co. Milwaukee, WIS, USA) enhanced staining with BCIP (5-bromo-4-chloro-3-indolyl-phosphate), NBT (4-nitroblue tetrazolium chloride) and levamisol was stopped when the blue precipitate was clearly visible. Sections were counterstained with Nuclear red solution and mounted with Euparal (ChromaGesellschaft, Stuttgart, Germany). Negative controls for *in situ* hybridization were performed using sense probes, which never gave any significant staining.

Whole mount in situ hybridization

Whole mount *in situ* hybridization was performed as described before (van Kleffens et al., 1998). Briefly, embryos were washed with PBS-Tween and treated with proteinase K (10 mg/ml; Boehringer Mannheim GmbH, Germany) for 15 minutes at room temperature. After 2 hours of pre-hybridization embryos were hybridized (250 ng/ml RNA probe) during 36 hours at 60°C in a hybridization mixture containing 50 % formamide, 4xSSC, 1xDenhardt's solution, herring sperm DNA (0.5 mg/ml), yeast tRNA (0.25 mg/ml), 10% dextran sulphate and heparin (50 mg/ml). The embryos were washed O/N at 60°C and blocked, then anti-DIG (1:2000) was added. Detection of hybridization signal took place by adding a detection buffer containing 100mM Tris-HCl (pH 9.5), 150 mM NaCl, 25 mM $MgCl_2$ and 0.3% Triton, followed by 2-6 hours incubation in the same buffer with 1 mM levamisol, 10% PVA (polyvinylalcohol, MW 31-50 kD, Aldrich Chemical Co. Milwaukee, WIS, USA), BCIP and NBT. The reaction was stopped by washing with TE and stained embryos were stored at 4°C in TE. Negative controls for whole mount in situ hybridization were performed using sense probes, which never gave any significant staining.

Immunohistochemistry

Sections were deparaffinized, washed with 100% ethanol and endogenous peroxidase was blocked (0.3% H_2O_2 in methanol, 15 minutes at room temperature). Depending on the antibody used, sections were either microwave treated or directly used for immunohistochemistry. The antibodies against human IGF-I, IGF-II and mouse type I IGF receptor (Research Diagnostics Inc., Flanders NJ, USA) were used in combination with microwave treatment (10 minutes boiling in citrate buffer pH8.5). The anti IGFBP-1 to -6 antibodies were applied directly, without pretreatment (van Kleffens et al., submitted). With all the antibodies an avidine-biotine complex (ABC) method was performed, except for the type I IGF receptor antibody, where a peroxidase-anti-peroxidase (PAP) method was used. Sections were blocked for endogenous biotine and avidine (Dako corporation, Carpinteria, CA, USA) and were incubated with 10% normal goat serum (IGFBP antibodies) or normal rabbit serum (anti IGF-I and -II antibodies). The anti-type I IGF receptor antibody was used in the PAP method, in which sections also were incubated with 10% normal goat serum. The serial sections were incubated overnight at 4∞C with the polyclonal anti-human IGF-I and -II and anti-mouse type 1 IGF receptor antibodies (1:100 in 3% BSA in PBS), or with the polyclonal antibodies against mouse IGFBP-1 to -6 (anti IGFBP-1 and -3 1:150 in 3% BSA in PBS, anti IGFBP-2, -4, -5 and -6 1:250 in 3% BSA in PBS). For the ABC method sequential incubations with a biotinylated goat anti-rabbit antibody (DAKO, Glostrup, Denmark) and the streptavidin horseradish peroxidase complex (Biogenex, San Ramon, CA, USA) were followed by development in 0.075% diaminobenzidine (DAB) in PBS containing 0.075% H_2O_2 for 4 minutes. DAB staining results in brown staining of immunoreactive cells. The PAP method consisted of sequential incubations with a rabbit anti-goat peroxidase conjugated antibody and rabbit PAP, followed by the DAB staining as described above. For both methods counterstaining was done with Mayers haematoxylin. Controls for type I IGF, IGF-I and IGF-II were performed by omitting the antibody from the immunohistochemical procedure. IGFBP staining patterns were analyzed in comparison to the respective pre-immune sera diluted 1:10.

Tunel

Tunel was used to detect apoptotic cells in tissue sections. This technique is based on the enzymatic labeling of the free 3' ends of the fragmented DNA of apoptotic cells. Specific detection of these labeled cells occurs by the use of antibodies.

Sections were deparaffinized and rehydrated, followed by incubation in DNase free Proteinase K (20µg/ml, Life Technologies, Breda, the Netherlands) during 15 minutes at 37°C. Endogenous peroxidases were removed with 2% H_2O_2 in PBS during 10 minutes at room temperature. The sequential sections were incubated with terminal deoxynucleotidyl transferase (TdT) buffer (0.5 M Cacodylate, 1 mM $CoCl_2$, 0.5 mM DTT, 0.15 M NaCl, 0.05% BSA; pH 6.8) for 5 minutes, followed by incubation with TdT enzyme (5U per slide; Promega, Madison, WIS, USA) and biotin-16-dUTP (0.5 nmol per slide; Boehringer Mannheim GmbH, Mannheim, Germany) in TdT buffer for 2 hours at 37°C. The reaction was stopped with TB buffer (300mM NaCl, 30 mM Na-Citrate) and sections were blocked with 3% BSA in PBS for 10 minutes before incubation with the streptavidin horseradish peroxidase complex for 30 minutes. Staining was done with DAB (as described above) and counterstaining was performed with Mayers haematoxylin. Controls were performed by omitting the TdT enzyme from the reaction mixture, which never gave any significant staining.

Nile Blue staining

To detect areas of cell death in the developing limb, embryos were stained directly after isolation from the mother with Nile Blue A (Sigma-Aldrich, Zwijndrecht, the Netherlands), a lysosomotropic vital dye. The number of granules stained blue by this dye corresponds to the number of clusters of dead cells phagocytized by macrophages (Kimura and Shiota, 1996). A water-saturated solution was diluted 1:500 in water. Vital embryos varying from 12.5 to 14.5 dpc were stained for 1 hour at room temperature. After washing 5 times in water limbs were analyzed directly or submerged in PBS at 4°C for one night.

Results

In this study we investigated mouse embryos of 9.5, 11.5 and 13.5 dpc. A detailed analysis is given of the localization of mRNA and protein expression of IGF system members at sites of active tissue remodeling, such as the eye, whiskers, nose, toothbud, ganglia, somites and limbs. Both IGFBP-1 and -6 mRNA and protein expression in all stages under study were below detection limits, in confirmation with former IGF-system gene expression studies of the developing mouse embryo (Schuller et al., 1993; Lindenbergh-Kortleve et al., 1997; van Kleffens et al., 1998) and are therefore not mentioned.

9.5 dpc

The results of *in situ* hybridization and immunohistochemistry are summarized in table 1.

In the optic vesicle type I IGF receptor (tIrec) was detected at the protein level but gene transcription could not be visualized. Furthermore, differences were found in mRNA expression and protein localization of IGF-I (only mRNA) and IGFBP-2 (only protein). Moreover, all IGF family members, except IGFBP-3, were localized in and/or around the one cell type layer of the optic vesicle (Fig. 1). In the 9.5 dpc. embryo most obvious apoptosis patterns occurred in and around (mesoderm) the optic vesicle (Fig. 1).

In the somites all IGF system members, except IGFBP-3, could be detected at mRNA and protein level, with one exception: of IGFBP-5 only mRNA was detected. Tunel results indicated that the somites, at this stage, did not yet show apoptosis.

Table 1

Localization of mRNA and protein of IGF system components and of apoptosis in 9.5 dpc structures of the mouse embryo

		optic vesicle	somites
IGF-I	mRNA	+	+
	protein		+
IGF-II	mRNA	+	+
	protein	±	+
tIrec	mRNA		+
	protein	+	+
IGFBP-2	mRNA		+
	protein	+	+
IGFBP-3	mRNA		
	protein		
IGFBP-4	mRNA	+	+
	protein	+	+
IGFBP-5	mRNA	+	+
	protein	+	
apoptosis		+	

Scoring
+ *signal is detected*
± *weak signal is detected*
blank *no signal could be detected*

Abbreviation
tIrec *type I IGF receptor*

11.5 dpc

At 11.5 dpc IGF system expression and apoptosis was correlated in eye, somites, ganglia, limb, nose and toothbuds. Table 2 summarizes the mRNA and protein localization of the IGF system components, together with the occurrence of cell death.

Expression of most IGF family members (both mRNA and protein) was detected in the eye, colocalized in the lens vesicle and the neural layer of the retina. An example of IGF-II in lens vesicle and neural retina is given in Figure 2a. The tIrec protein was expressed in the lens vesicle, but could not be detected in the neural layer. In the presumptive pigmented layer of the retina only IGFBP-2 was weakly expressed. For IGFBP-3 only mRNA expression was visualized in the lens vesicle and neural retina, as well as in the other regions analysed. mRNA of the tIrec could be detected in the developing eye muscles. The other IGF system members did not show any mRNA or protein staining in this tissue. In the eye, apoptotic cell death occurred in the lens, and in some cells of the neural layer of the retina, but not in the eye muscles (Fig. 2b).

IGF-II (protein only), tIrec and IGFBP-2 were detected in the early stage of olfactory bulb formation. Also in this region, apoptotic cells were found.

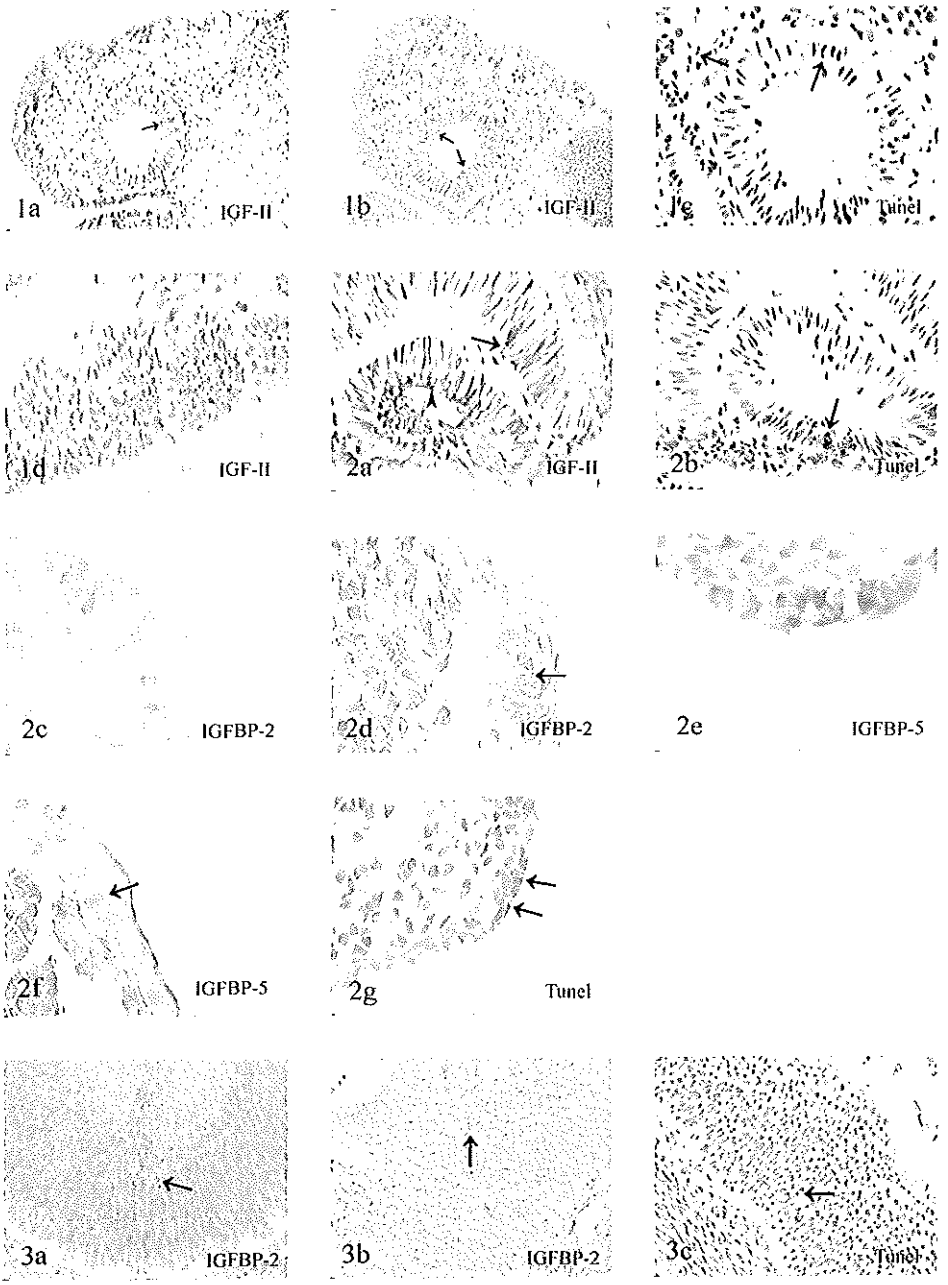
In the early stage of toothbud formation, only IGFBP-5 mRNA could be detected. mRNA signal was noticed in the dental lamina, where also Tunel positive cells were found.

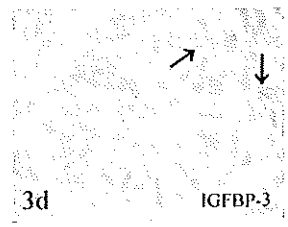
At 11.5 dpc early stage whisker formation has started. The formed whisker placode showed signal for the type I IGF receptor and IGF-I (mRNA). Cell death was limited in this early stage of whisker formation and was confined to the central part of the whiskers, as confirmed by Nile Blue staining of whole embryo's (see Discussion, Figure 4).

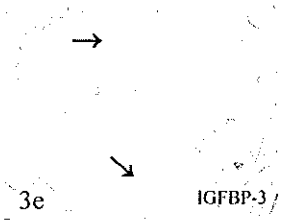
Expression in the apical ectodermal ridge (AER) was only seen for IGFBP-2 and -5 (mRNA only). These expression patterns colocalized with patterns of apoptotic cell death. Protein localization in limb was identical to the mRNA patterns (Fig. 2c-g).

Expression in the ganglia occurred for all members of the IGF system, except IGFBP-5. All patterns were identical, consisting of various cells of the same type, homogeneously distributed throughout the ganglion. This was similar to the apoptotic cell death pattern.

In the somites expression of IGF-I and the type I IGF receptor had ceased at 11.5 dpc and only IGF-I protein was found both in and around the sclerocoel (centre). IGFBP-2 mRNA and protein were located in and around the sclerocoel. Around the sclerocoel, IGFBP-3 (only mRNA), -4 and -5 were located. Again, no signal was obtained in somites after Tunel staining.


1a
IGF-II
1b
IGF-II
1c
Tunel
1d
IGF-II
2a
IGF-II
2b
Tunel
2c
IGFBP-2
2d
IGFBP-2
2e
IGFBP-5
2f
IGFBP-5
2g
Tunel
3a
IGFBP-2
3b
IGFBP-2
3c
Tunel


3d
IGFBP-3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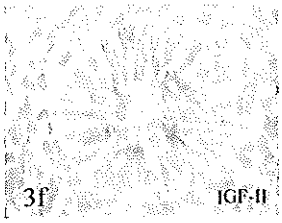
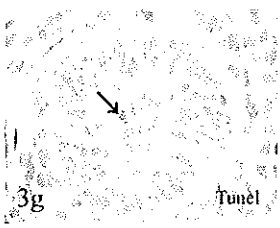
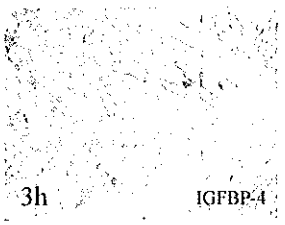
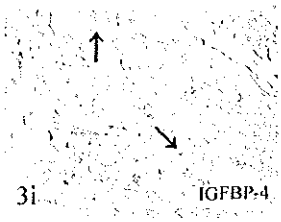
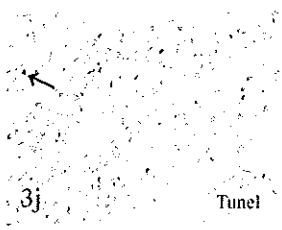
IGFBP-3

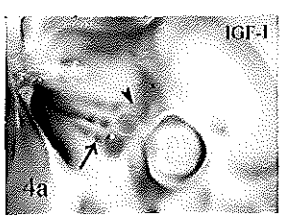

3f
IGF-II

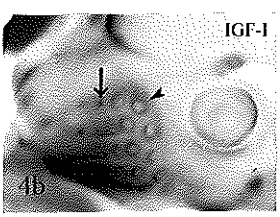

3g
Tunel

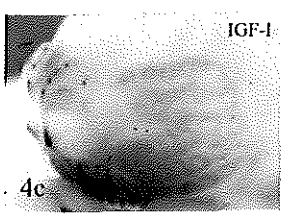

3h
IGFBP-4

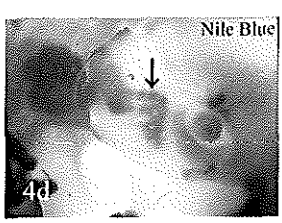

3i
IGFBP-4

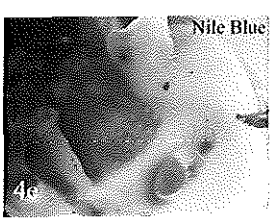

3j
Tunel


IGF-I
4a


IGF-I
4b


IGF-I
4c


Nile Blue
4d


Nile Blue
4e

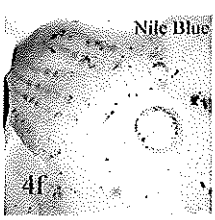

Nile Blue
4f

Figure 1
Analysis of IGF system mRNA (blue) and protein (brown) expression and apoptosis patterns (brown) in sagittal sections of 9.5 dpc mouse embryos. a: IGF-II mRNA in the optic vesicle (arrow). b: IGF-II protein in the optic vesicle (arrows). c: apoptotic cells in the optic vesicle (arrows). d: IGF-II mRNA in the somites.

Figure 2
Analysis of IGF system mRNA (blue) and protein (brown) expression and apoptosis patterns (brown) in sagittal sections of 11.5 dpc mouse embryos. a: IGF-II mRNA in the lens vesicle (arrowhead) and neural retina (arrow). b: apoptotic cells in the lens vesicle (arrow). c: IGFBP-2 mRNA in the AER. d: IGFBP-2 protein in the AER (arrow). e: IGFBP-5 mRNA in the AER. f: IGFBP-5 protein in the AER (arrow). g: apoptotic cells in the AER (arrows).

Figure 3
Analysis of IGF system mRNA (blue) and protein (brown) expression and apoptosis patterns (brown) in sagittal sections of 13.5 dpc mouse embryos. a: IGFBP-2 mRNA in neural retina (arrow). b: IGFBP-2 protein in the neural retina (arrow). c: apoptotic cells in the neural retina (arrow). d: IGFBP-3 mRNA in whisker (arrows). e: IGFBP-3 protein in whisker (arrows). f: IGF-II mRNA in whisker. g: apoptotic cells in whisker (arrow). h: IGFBP-4 mRNA in thoracic ganglion. i: IGFBP-4 protein in thoracic ganglion (arrow). j: apoptotic cells in thoracic ganglion (arrow).

Figure 4
Lateral view of whole mount in situ mRNA hybridization (dark blue/black) and Nile Blue staining (dark blue) in mouse embryos at various stages of development. a-c and d-f show the difference in timing between IGF-I mRNA expression and occurrence of apoptotic cell death in the developing whiskers. a: IGF-I mRNA in the whiskers of a 11.5 dpc mouse embryo. Arrow: newly formed whiskers show signal in the centre. Arrowhead: earlier existing whiskers show signal around the whiskers. b: IGF-I mRNA in the whiskers of a 12.5 dpc mouse embryo. Arrow: newly formed whiskers show signal in the centre. Arrowhead: earlier existing whiskers show signal around the whiskers. c: IGF-I mRNA around the whiskers of a 13.5 dpc mouse embryo. d: Apoptotic cell death patterns in the whiskers of a 11.5 dpc embryo. Arrow: difference with IGF-I staining is that now the whole whisker is stained, and not only the centre, of the newly formed whiskers. e: Apoptotic cell death patterns in the whiskers of a 12.5 dpc embryo. f: Apoptotic cell death patterns in the whiskers of a 13.5 dpc embryo.

Table 2

Localization of mRNA and protein of IGF system components and of apoptosis in 11.5 dpc structures of the mouse embryo

		lens vesicle	retina-neural layer	retina-pigm.layer	eye muscle	olfactory bulb	toothbud	whiskers	limb-mesoderm	limb-AER	ganglia	somites-sclerocoel	somites-around sclerocoel
IGF-I	mRNA	+	+						+		+	+	
	protein	+	+						+		+	+	+
IGF-II	mRNA	+	+						+		+	+	
	protein	+	+			+			+		+	+	
tIrec	mRNA	+	+		+	+		+	+		+		
	protein	+				+			+		+		
IGFBP-2	mRNA	+	+	±		+			+	+	+	+	±
	protein	+	+	±		+			+	+			
IGFBP-3	mRNA	+	±								+		+
	protein												
IGFBP-4	mRNA	+	+								+		+
	protein	+	+								+		+
IGFBP-5	mRNA						+			+			+
	protein												+
apoptosis		+	+			+		+		+	+		

Scoring
+ *signal is detected*
± *weak signal is detected*
blank *no signal could be detected*

Abbreviation
tIrec *typeI IGF receptor*
AER *apical ectodermal ridge*

13.5 dpc

In the 13.5 dpc embryo all the major structures are already visible. IGF system expression (mRNA and protein) and apoptosis in eye, nose, toothbuds, whiskers, limb, ganglia and somites were analyzed. The results are summarized in Table 3.

In the lens, all IGF system components were detected at the mRNA as well as the protein level, with two exceptions. IGF-II protein and IGFBP-5 mRNA could not be detected in the lens. In the neural layer of the retina most IGF system components studied, except IGFBP-3 and -5, were found. IGFBP-2 expression in the neural retina is shown in Figures 3a and 3b. Only IGF-I could not be detected simultaneously at mRNA and protein level. In the retinal inner layer also apoptotic cells were found (Fig. 3c). Whereas in the pigmented layer of the retina only IGFBP-2 mRNA and protein and IGFBP-5 mRNA were detected and no apoptotic cell death was noticed.

In the eye muscles, IGF-II, type I IGF receptor, IGFBP-2, -3 and -5 were detected, both at the mRNA and protein levels. In contrast to the 11.5 dpc eye muscles, apoptotic cells were detected at 13.5 dpc.

In the olfactory bulb we found mRNA and protein of IGF-II, IGFBP-2 and IGFBP-4. Only IGF-I protein was detected, whereas for the type I IGF receptor only mRNA was detected. Here we also found a limited number of apoptotic cells. Another part of the nose, around the nostrils (nasal aparture), showed more apoptotic cells. In this region we only detected IGF-II and IGFBP-2 mRNA and protein, and IGFBP-5 mRNA.

In the toothbuds, all the IGF system components, except IGFBP-4, were localized. Also Tunel staining showed positive cells in this region.

In the whiskers all IGF system members were localized, especially in the outer rim (the whisker follicle). Figures 3d and 3e show IGFBP-3 mRNA and protein in the outer rim of the whisker. In this area IGF-II and IGFBP-4 mRNA were not detected. In the central part of the whiskers, the primordium of the dermal papilla (called inner rim in Table 3), IGF-II (Fig. 3f), type I IGF receptor, IGFBP-2 and -4 were expressed at the mRNA and at the protein level. IGF-I could only be detected at the protein level. Tunel staining occurred just in a few apoptotic cells of both the whisker follicle and the primordium of the dermal papilla (Fig. 3g).

Also in the limb very specific IGF system and cell death patterns were found. In the precartilage only IGF-I and IGF-II mRNA and type I IGF receptor mRNA and protein were detected.

In the interdigital zone (IDZ) IGFBP-2, 4 and 5 were localized, mRNA as well as protein. IGFBP-3 was only found at the mRNA level in the IDZ. In the ectoderm expression was limited to IGFBP-2 (mRNA and protein). In the mesoderm surrounding the precartilage IGFBP-2 mRNA was observed, whereas for IGF-I, IGF-II and IGFBP-5 only protein was detected in this part of the limb. In this region no staining of apoptotic cells could be noticed.

In the ganglia many IGF system components were localized at mRNA and protein level. However, IGFBP-3 mRNA and IGFBP-5 protein were not visualized, while IGFBP-2 was not detected at all. The pattern of the expressed IGF system members and the pattern of cell death were similar, comparable with the 11.5 dpc situation (Fig. 3h-j).

Localization of the IGF system genes in the somites was limited to mRNA of the type I IGF receptor and IGFBP-2 (both in and around the sclerocoel) and mRNA and protein of IGFBP-4 and -5 (around the sclerocoel). In addition, only at this stage of development apoptotic cells were detected in the somites (in and around the sclerocoel).

Table 3

Localization of mRNA and protein of IGF system components and of apoptosis in 13.5 dpc structures of the mouse embryo

		lens	retina-neural layer	retina-pigm.layer	eye muscle	nose-olfactory bulb	nose-nostril	toothbud	whisker-outer rim	whisker-inner rim	limb-precartilage	limb-IDZ	limb-ectoderm	limb-mesoderm	ganglia	somites-sclerocoel	somites-around sclerocoel
IGF-I	mRNA	+						+	+		+				+		
	protein	+	+			±		+	+	+				+	+		
IGF-II	mRNA	+	+		+	+	±	+		+	+				+		
	protein		+		+	+	+	+	+	+				+	+		
tIrec	mRNA	+	+		+	+		+	+	+	+				+	+	+
	protein	+	+		+			+	+	+	+				+		
IGFBP-2	mRNA	+	+	±	+	+	+	+	+	+		+	+	+		+	±
	protein	+	+	+	+	+	+	+	+	+		+	+				
IGFBP-3	mRNA	+			±			+	+			+					
	protein	±			±			+	+						+		
IGFBP-4	mRNA	+	+			+				+		+			+		+
	protein	+	+			+			+	+		+			+		±
IGFBP-5	mRNA			+	+		+	+	+			+			+		+
	protein	+			+			+	+			+		+			+
apoptosis		+	+		+	±	+	+	±	±		+			+	+	+

Scoring
+ *signal is detected*
± *weak signal is detected*
blank *no signal could be detected*

Abbreviation
tIrec *typeI IGF receptor*
AER *apical ectodermal ridge*

Discussion

In a previous study we showed the differential and spatio-temporal specific mRNA expression patterns of the IGF system in the developing mouse limb (van Kleffens et al. 1998). In addition, we demonstrated colocalization of specific IGF system members with cell death regions in the developing limb. In the present study, we correlated mRNA and protein expression of the IGF system components in the mouse embryo, during various stages of development, using recently generated specific polyclonal antibodies to mIGFBP-1 through -6 (van Kleffens et al., submitted). Regions that show apoptosis during specific stages of development were analyzed. To our knowledge, this is the first study that links mRNA and protein data of the IGF system with apoptosis patterns during mouse development.

Our results confirm the differential spatio-temporal mRNA expression patterns of the IGF system during mouse development (Schuller et al., 1993; Lindenbergh-Kortleve et al. 1997; van Kleffens et al., 1998). Furthermore, we show that protein localization of the IGF system is differential and tissue-specific as well.

As expected, mRNA and protein localization of the IGF system components did not completely overlap. In general, in all developmental stages analyzed in this study, mRNA and protein localization of the type I IGF receptor, IGFBP-2 and IGFBP-4 showed highest degree of correlation. As the type I IGF receptor is membrane bound, this correlation was to be expected. For IGFBP-2 and -4, that are secreted proteins, cell attachment has not been described, although IGFBP-2 has a RGD site (Jones and Clemmons 1995).

Our data show that the localization of IGF system proteins can not be predicted by corresponding mRNA expression patterns. This indicates that IGFBPs are not necessarily active at their mRNA expression sites. Through transportation to specific tissues and organs an additional mechanism for tissue-specific regulation of IGF bioactivity by the IGFBPs may be provided.

In support of presumed IGF-independent actions (IGFBPs function without using mediation by IGF- type I IGF receptor) of the IGFBPs (Oh et al., 1993, Kim et al., 1997) IGFBP expression was found in tissues or cell types where no type I IGF receptor was expressed, such as the presumptive pigment layer of the retina, nostrils, and parts of the developing limb.

Close contact between the neuroepithelium of the optic vesicle and the overlying surface ectoderm guides the multistep process of lens induction (Graw, 1996). The optic vesicle is thought to provide a diffusible factor, that induces lens determination (Piatigorsky, 1981). IGFs are known to stimulate elongation of lens fibres (Beebe et al., 1980, 1987). Here at 9.5 dpc IGF-I and -II and multiple IGFBPs were detected in the optic vesicle, suggesting a possible function of the IGF system already at a preceding stage, i.e. during lens induction. The observed apoptotic cells in the mesoderm between the optic vesicle and presumptive lens may provide a possible mechanism to bring about these structures.

As to the suggested protective function of the IGF system in apoptosis (Resnicoff et al., 1995a/b; Chun et al., 1994; Ueda and Ganem, 1996; Christofori et al., 1994), our results show that localization of the IGFs, the type I IGF receptor and IGFBP-2 correlates well with cell death regions. In general, when these genes were expressed no apoptotic cells could be detected, and vice versa. Correlation of IGFBP-3, -4 and -5 expression with cell death regions becomes evident only at 13.5 dpc. This may indicate that these IGFBPs function to trap IGFs, thereby stimulating apoptosis. This is in accordance with the

reported involvement of the IGFBPs in apoptosis in both *in vitro* and *in vivo* experiments (Tonner et al., 1995; Damon et al., 1998; Nickerson et al., 1998).

Of specific interest is the expression pattern of IGF-I in the developing whiskers. IGF-I is present in the developing whiskers at 11.5 dpc and fades at 12.5 dpc. At 13.5 dpc IGF-I expression is gone, whereas apoptosis occurs when IGF-I expression decreases. This may indicate a protective function of IGF-I against apoptosis in the developing whisker. This was clearly visualized by whole mount *in situ* hybridization and Nile Blue staining and is depicted in Figure 4.

Similarly, type I IGF receptor expression in the eye muscles precedes the onset of apoptosis. In contrast, here apoptosis occurs without a decrease of type I IGF receptor expression. This may indicate that the presence of type I IGF receptor is protective against apoptosis and that the inhibiting effect of IGF action mediated by the type I IGF receptor in the 13.5 dpc eye muscles is overruled by the action of other IGF system components.

At 13.5 dpc less IGF system members are expressed in the somites than in the 11.5 dpc embryo, while the number of apoptotic cells is increased at 13.5 dpc in the somites. This may be another indication for a role of the IGF system in protection against apoptosis.

In conclusion, we have localized mRNA and protein expression of the IGF system and apoptosis in 9.5 to 13.5 dpc mouse embryos. Expression patterns of the IGFs, type I IGF receptor and IGFBP-2 correspond well with the occurrence of apoptotic cell death in the developing mouse embryo. Furthermore, an important finding is that mRNA expression patterns of the secreted IGF system components do not necessarily predict protein localization in the mouse embryo. Our results confirm the described inhibiting function in apoptosis of the IGFs and the type I IGF receptor. We suggest that tissue-specific modulation of IGF protective action by the different IGFBPs during mouse embryonal development may contribute to the characteristic morphological changes.

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4

mRNA and protein localization of the IGF system during mouse embryonic development in areas with apoptosis

Chapter 5

Human IGFBP-1 inhibits IGF-I stimulated body growth but stimulates growth of the kidney in Snell dwarf mice

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Abstract

The actions of insulin-like growth factor-I (IGF-I) are modulated by IGF-binding proteins. The effects of IGFBP-1 *in vivo* are not sufficiently known, with respect to inhibitory or stimulatory actions on IGF-induced growth of specific organs. Therefore, we studied the effects of IGFBP-1 on IGF-I-induced somatic and organ growth in pituitary deficient Snell dwarf mice, which lack endogenous GH and IGF-I production. Human growth hormone (hGH), IGF-I, IGFBP-1 and a pre-equilibrated combination of equimolar amounts of IGF-I and IGFBP-1 were administered subcutaneously (age mice 6-8 weeks) during 4 weeks, in two independent experiments.

Treatment with IGF-I alone induced a significant increase in body length and weight, and an increase in weight of the submandibular salivary glands, kidneys, femoral muscles, testes and spleen. Liver, brain, heart and thymus were not significantly affected. IGFBP-1 alone induced a significant increase in weight of the kidneys. In contrast, growth of the thymus was significantly inhibited. Co-administration of IGF-I with IGFBP-1 neutralized the stimulating effects of IGF-I on body length and weight as well as on the femoral muscles and testes, but not on the submandibular salivary glands, kidneys and spleen.

IGFBP-I concentration in pooled sera collected two hours after the last injection were higher after administration of IGFBP-I alone than in combination with IGF-I, indicating a slower clearance from the circulation in the absence of IGF-I. The presence of injected IGFBP-1 in the sera of the mice treated with IGFBP-1 alone and with the complex, was confirmed by immunoblotting. Western ligand blotting showed induction of the IGFBP-3 doublet by IGF-I alone and in combination with IGFBP-1, and to a minor extent by treatment with hGH. A similar pattern was obtained for IGFBPs with molecular weights of 24 kD, most probably IGFBP-4.

Our data show that co-administration of IGFBP-1 differentially inhibits IGF-I-induced body and organ growth in GH-deficient mice. In this respect, the remarkable additive and stimulating effects of IGF-I and IGFBP-1 on kidneys, submandibular salivary glands and spleen are noteworthy. These data warrant further investigation, since elevated levels of IGFBP-1 are associated with several disease states and such a higher level of IGFBP-1 may influence IGF-I-induced anabolic processes.

Introduction

It is generally proposed that IGFBP-1 to -6 and IGFBP-proteases modulate the tissue availability of the IGFs (1-4). Amongst them IGFBP-1 plays an important role in glucose homeostasis in which insulin is the major modulator (5). IGFBP-1 in serum is mainly uncomplexed to IGFs, in contrast to IGFBP-3, which is the principal carrier of the IGFs in serum. Especially in several disease states, such as in GH-deficiency, type I diabetes and during fasting, IGFBP-1 in serum is elevated and in these cases IGFBP-1 has been postulated as an important modulator of free IGF levels together with IGFBP-2 (3,5). IGFBP-1 is phosphorylated (6,7) and *in vitro* studies suggest that phosphorylation of IGFBP-1 is associated with an increased affinity for IGFs (8) and nonphosphorylated IGFBP-1 seems to be less inhibitory for IGF actions than the phosphorylated form (9).

The physiological role of IGFBP-1 *in vivo* is still unclear. It has been shown that recombinant nonphosphorylated IGFBP-1 inhibits growth stimulation by IGF-I and GH in hypophysectomized rats (10) and co-administration of amniotic fluid-derived hIGFBP-1, in which the nonphosphorylated form predominates, inhibited IGF-I-induced hypoglycemia in rats (11,12).

Also in transgenic mice overexpressing IGFBP-1, somatic growth retardation has been observed (13-15). In all these studies a molar excess of IGFBP-1 above IGF-I was present in serum. These data strengthen the view that IGFBP-1 functions primarily as a competitor of IGF receptors.

In vivo studies showing potentiation of IGFs by IGFBP-1 are limited to the promotion of wound healing by local application to skin incisions in rats (16) and to dermal ulcers in both normal rabbits and in rabbits with diabetes (17). Results were dependent of the molar ratio of IGF-I to IGFBP-1 used and of the phosphorylation state of IGFBP-1.

Little is known, however, about the effects of IGFBP-1 on IGF-I-induced organ growth in GH-deficiency. This is of importance since elevated levels of serum IGFBP-1 in several disease states may influence IGF-I-induced anabolic processes. Therefore we investigated the effects of amniotic fluid-derived hIGFBP-1 alone and in combination with IGF-I (1:1 molar ratio) on total body growth and growth of specific organs in the Pit-1 deficient Snell dwarf mice (18). In addition, serum IGFBP-1 and IGF-I antibody formation and serum IGFBPs profiles were investigated in order to obtain more insight into the observed growth responses.

Materials and methods

Test substances

E.coli-derived recombinant human IGF-I (IGF-I) was kindly provided by Eli Lilly Co. (Indianapolis, IN). Recombinant human growth hormone (hGH) was from Kabi-Pharmacia (Uppsala, Sweden).

Human IGFBP-1 was purified from amniotic fluid from pregnant women (midterm) which was obtained for diagnostic purposes and approved by the ethical committee. Amniotic fluid was filtrated, followed by an ammonium sulfate precipitation (1.8M) of the supernatant overnight at room temperature. After centrifugation, the pellet was washed, centrifuged again and dissolved in water. Precipitation occurred in methanol at a final concentration of 65%. After 30 min at room temperature the solution was centrifuged. The resulting supernatant was separated on a C18 column. The IGFBP-1 fractions were

pooled and precipitated with 0.6 volume aceton overnight at -20°C. After centrifugation of the aceton precipitate the pellet was dissolved in 10 mM NH_4HCO_3 (pH 7.5) and vacuum dried. The resulting hIGFBP-1 had a purity of 95%.

Animals and experimental design

Snell dwarf mice were bred and kept under standardized laboratory conditions as described earlier (19). Groups consisted of males and females and were selected such as to obtain equal means and standard deviations for length and weight. hGH, IGF-I and IGFBP-1 were dissolved in phosphate buffered saline (PBS), pH 7.4. For the co-administration experiments, IGF-I and IGFBP-1 were incubated overnight in equimolar amounts in PBS (pH 7.4) at 40°C (20). The solutions were used for 4 weeks and stored in the dark at -20°C.

In two independent long-term experiments dwarf mice (age 6-8 weeks at the start of the experiment) received hGH (16.6 mU/day), IGF-I (30 (µg/day) (18), IGFBP-1 (105 (µg/day), IGF-I+IGFBP-1 (30+105 (µg/day) or PBS during 4 weeks. In order to avoid hypoglycemia 10% glucose was added to the drinking water, starting one week before the experiment. Dwarfs (n=5 per group in each experiment) were injected subcutaneously in the neck with 0.1 ml hormone solution or vehicle, three times daily, 5 days a week. All animals were weighed and the total length was measured under ether anaesthesia once a week by the method of Hughes and Tanner (21). The animals were killed by decapitation 2h after the last injection. Blood was collected and the sera were pooled per group in order to collect sufficient amounts for all serum determinations (see below) and stored at -20°C. Organs were removed and weighed to the nearest milligram.

Serum determinations

Serum IGF-I and IGF-II were routinely measured by heterologous radioimmunoassays (RIA) after acid Sep-Pak C18 (Water Associates Milford, MA, USA) chromatography as described previously (22).

Human IGFBP-1 levels were determined by RIA using IGFBP-1 purified from human amniotic fluid and a mouse monoclonal antibody against hIGFBP-1 (23). Displacement studies with purified hIGFBP-1 or serum indicated that ^{125}I-labeled-hIGFBP-1 (as prepared by either the chloramine-T, iodogen or lactoperoxidase method) was not suitable as a tracer. Instead, we used a covalent complex of native ^{125}I-labeled-hIGF-I and hIGFBP-1 which was prepared essentially according to the method described by Baxter and Martin (24). Unreacted iodo-IGF-I was separated from the ^{125}I-labeled-hIGF-I-IGFBP-1 complex by successive adsorption to charcoal at neutral and acid pH (pH 3.0), respectively. An excess of hIGF-I was added to the assay mixture (see below) in order to prevent possible interference from free ^{125}I-labeled-hIGF-I contaminating the radioactive complex in the RIA. The RIA buffer was composed of 0.1 M sodium phosphate (pH 6.5), 0.05% (w/v) Tween-20, 0.2% BSA and 0.02% NaN_3. Standards were prepared from purified hIGFBP-1 and stored at -70°C. Standard dilutions ranged from 0.19-15 ng per tube. Duplicates of serum samples were diluted 1:3 with assay buffer. The incubation mixture consisted of 100 µL assay buffer, 50 µL standard or diluted sample, 50 µL IGF-I solution (100 ng/mL), 50 µL antibody (1:650,000), and 50 µL tracer (10,000 cpm). After incubation for 18 hr at 4°C in polystyrene tubes, 100 µL Sac-Cel solid phase anti-mouse coated cellulose suspension (IDS, Boldon, England) was added. Complexation was complete after 30 min

at 20°C and 0.6 ml distilled water was added to the samples which were subsequently centrifuged at 1000 x g for 3 min. Pellets were washed once with 0.6 ml distilled water and counted. The sensitivity of the assay was approximately 1 ng/mL. The intra-assay variation was 7.9% at 31.6 ng/mL and 12% at 8.7 ng/mL. The inter-assay variation was 10% at 27.4 ng/mL.

In order to rule out the possibility of antibody formation to IGF-I or IGFBP-1 during treatment of the dwarf mice, sera were incubated with ^{125}I-labeled-IGFBP-1 or ^{125}I-labeled-IGF-I at different dilutions, followed by precipitation by either a polyethyleneglycol mixture (Immuno Nuclear Corp.) in the case of IGF-I, or the Sac-Cel solid phase anti mouse/rat suspension in the case of IGFBP-1. The incubation conditions were similar to those described for the regular RIAs for IGF-I and IGFBP-1, respectively (22).

Serum IGFBPs were analyzed by Western ligand blotting according to Hossenlopp (25), as described previously (22). hIGFBP-1 in the sera was analyzed by Western immunoblotting using the mouse monoclonal IGFBP-1 antibody described above and visualized by enhanced chemiluminescence (ECL), (Amersham International, Buckinghamshire, England). In the immunoblot procedure the primary antibody was diluted 1:8,000, while the second antibody, goat-anti-mouse IgG conjugated with peroxidase, was diluted 1:10,000.

IGFBP-3 proteolysis was determined by the IGFBP-3 protease assay as described previously (26). Quantification of the gels was performed by phosphorimaging using a GS-363 Molecular Imager System with Molecular Analyst software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Blood glucose levels were measured in each animal using a Lifescan One-touch II glucose analyzer (Johnson and Johnson).

Statistical analysis

Student's *t*-test and the S-method described by Scheffé (Scheffé, 1959) were applied in order to test for differences between groups.

Results

Body growth

The effects on body growth of Snell dwarf mice treated with IGF-I and IGFBP-1 alone or in combination were tested in two separate but identically designed experiments and compared with hGH treatment. Since the results obtained in both experiments were similar, data were pooled unless indicated otherwise. As shown in Fig.1, IGF-I stimulated total body length and weight significantly, confirming our previously published data (19,22). IGFBP-1 alone did not affect body length, whereas a slight non-significant decrease was observed for body weight. IGFBP-1 in combination with IGF-I inhibited the IGF-I-induced growth stimulation. hGH, at the dose tested, was more stimulatory than IGF-I.

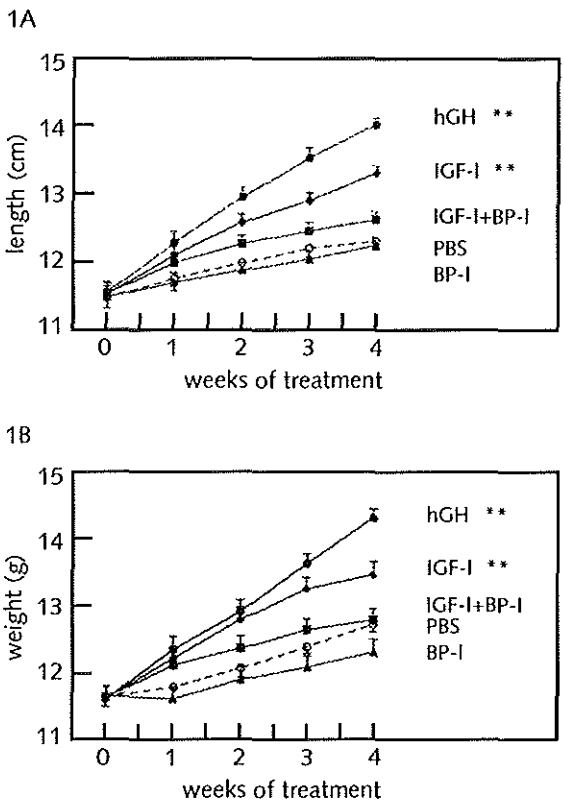


Figure 1
Body length (upper panel) and body weight (lower panel) of dwarf mice during treatment with PBS (O-O), IGF-I 30 (µg/day; ◆-◆), IGFBP-1 (105 µg/day; ▲-▲), IGF-I+IGFBP-1 (30+105 µg/day; ■-■) and hGH (16.6 mU/day; ●-●). The mean ± SEM is given. Number of animals for all treatments n=10. Significant differences (P<0.05) compared to PBS treated controls after 4 weeks of treatment were determined by Scheffé's test (**) or Student's t-test (*).

Growth of individual organs

Fig.2 shows body length and weight as well as the weights of several organs expressed as a percentage of the controls after 4 weeks of treatment. Significant differences between the groups are given in Table 1. Similar to hGH, IGF-I stimulated growth of the muscles quadriceps femoris and the testes, whereas IGFBP-1 alone had no effect. As for body length and body weight, co-injection of IGFBP-1 and IGF-I abolished the growth stimulatory effect of IGF-I on growth of these organs. As observed for muscles and testes, the weights of the kidneys, the submandibular salivary glands and the spleen significantly increased by treatment with hGH and IGF-I compared to controls. For the latter three organs, however, IGFBP-1 alone was stimulatory, although only for the kidneys significance was reached. The remarkable effects on the kidneys obtained with IGFBP-1 were also significantly higher than with IGF-I alone (Table 1). The combination of IGF-I and IGFBP-1 shows additive effects with respect to weight increase of kidneys, spleen and submandibular salivary glands compared to the effects obtained with both peptides administered alone (Fig.2, Table 1).

In contrast to hGH, IGF-I did not stimulate growth of the thymus, in accordance with data published before (27). IGFBP-1 alone or in combination with IGF-I, however, caused a significant decrease in weight of the thymus. Weights of the brain and the liver were unaffected by either IGF-I and IGFBP-1 alone, or by the combination of both peptides (data not shown).

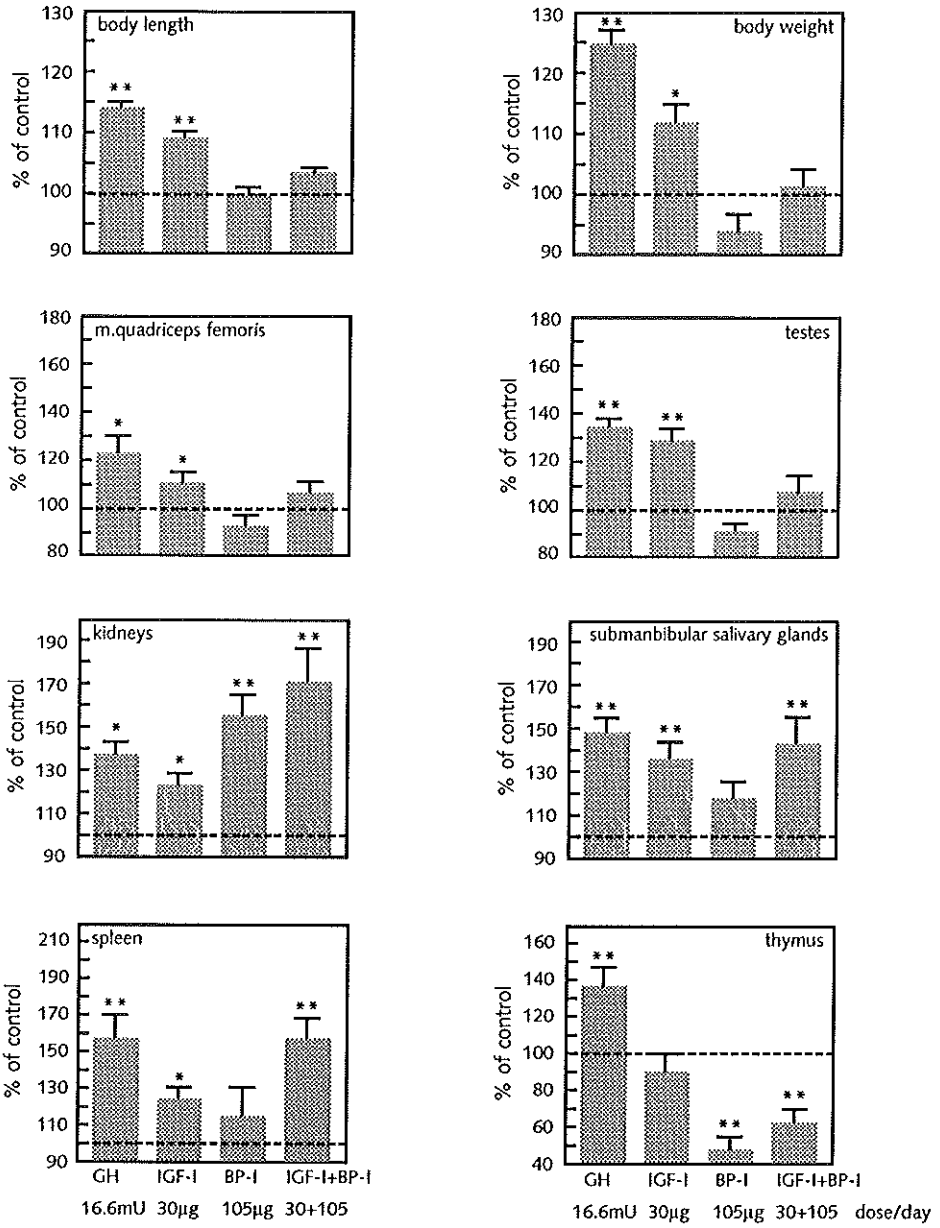


Figure 2
Organ growth of dwarf mice after 4 weeks of treatment with PBS, hGH, IGF-I, IGFBP-1, and IGF-I+IGFBP-1, expressed as a percentage of the PBS treated controls. For details, see Fig. 1 and the Materials and Methods section. Number of animals for all treatments n=9-10, except for the testes n=4-6. Significant differences compared to PBS treated controls were determined by Scheffé's (**) or Student's t-test (*).

Table 1

Statistical differences ($P \leq 0.05$) between the different treatments (after 4 weeks) as tested with Student's t-test (*) or Scheffé test (**)

Treatment groups compared	Body length	Body weight	M. quadriceps femoris	Testes	Kidneys	Submandibular salivary glands	Spleen	Thymus
hGH vs. IGF-I	**	**	n.s.	n.s.	n.s.	n.s.	*	**
IGF-I vs. IGFBP-I	**	**	*	**	*	n.s.	n.s.	**
IGF-I vs. IGF-I+IGFBP-I	**	*	n.s.	*	**	n.s.	*	*
IGFBP-I vs. IGF-I+ IGFBP-I	n.s.	n.s.	n.s.	*	n.s.	n.s.	*	n.s.

n.s. *non significant*

Serum glucose, IGFs and IGFBPs

Serum glucose levels were significantly decreased by IGF-I alone, compared to the controls. hGH and IGFBP-1 alone did not influence serum glucose levels (Table 2). IGFBP-1 in combination with IGF-I inhibited the IGF-I-induced hypoglycemia.

Serum hIGFBP-1 levels in both experiments were higher after administration of IGFBP-1 alone than after treatment with the combination of IGF-I and IGFBP-1 (Table 2). The relatively high value measured in PBS-treated mice is likely due to interference with mouse γ-globulins, since a mouse-monoclonal was used. Absorption of serum with protein A Sepharose abolished this interference, while "spiking" of this stripped serum with hIGFBP-1 resulted in a recovery of 100%. The presence of hIGFBP-1 was confirmed by Western immunoblotting (data not shown). Serum IGF-I concentrations were markedly increased after injection of IGF-I, whereas only a slight rise was observed after hGH administration (Table 2). IGF-I values of the other treatment regimes were unreliable, caused by interference of IGFBP-1 in the IGF-I radioimmunoassay (data not shown).

The inhibition of IGF-I-induced growth by IGFBP-1 of total body growth and growth of distinct organs cannot be explained by antibody formation against either IGF-I or IGFBP-1. Antibodies against IGFBP-1 could be detected only at very low levels after 4 weeks of treatment with IGFBP-1 alone or in combination with IGF-I. Antibodies against human IGF-I in the IGF-I treated mice were undetectable.

Western ligand blotting of the sera, using ^{125}I-labeled IGF-II as tracer, showed induction of the IGFBP-3 doublet (mol.weight 42 and 38 kD). This was more pronounced in the group treated with IGF-I or IGF-I+IGFBP-1 than in the group treated with hGH. IGFBP-1 alone had no effect and did not influence the IGF-I-induced IGFBP-3 expression in serum (Fig.3A and B).

IGFBP-1 was present in high amounts at mol.weight 30 kD, confirming the radioimmunoassay data. With respect to the 24 kD band, most probably representing IGFBP-4, a marginal increase was observed of the intensity after treatment with hGH and even more after administration of IGFBP-1, IGF-I and the combination of IGF-I+IGFBP-1.

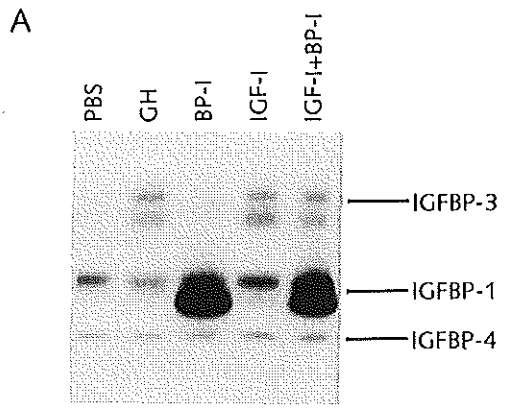
IGFBP-3 proteolysis was slightly decreased by hGH (26), and in the same order of magnitude by IGF-I alone or in combination with IGFBP-1 (Fig.4). IGFBP-1 did not influence this activity. Inhibition by EDTA was slightly better in the hGH, IGF-I and IGF-I+IGFBP-1 treated mice than in the PBS and IGFBP-1 treated animals.

Table 2
Serum parameters after 4 weeks of treatment

Treatment	blood glucose [1] mmol/l	IGF-I [2] ng/ml	hIGFBP-1 [2] µg/ml
PBS	8.0 ± 0.3	13.4	0.5
hGH	9.1 ± 0.4	23.2	n.t.
IGF-I	5.7 ± 0.7**	417.2	n.t.
IGFBP-I	8.4 ± 0.3	~	12.0
IGF-I + IGFBP-1	6.9 ± 0.6	~	4.1

Significant differences ($P \leq 0.05$) were tested by Student's *t*-test (*) or Scheffé test (**)

n.t. *not tested*
~ *not measurable*
[1] *number of animals: n=10*
[2] *mean of two experiments*



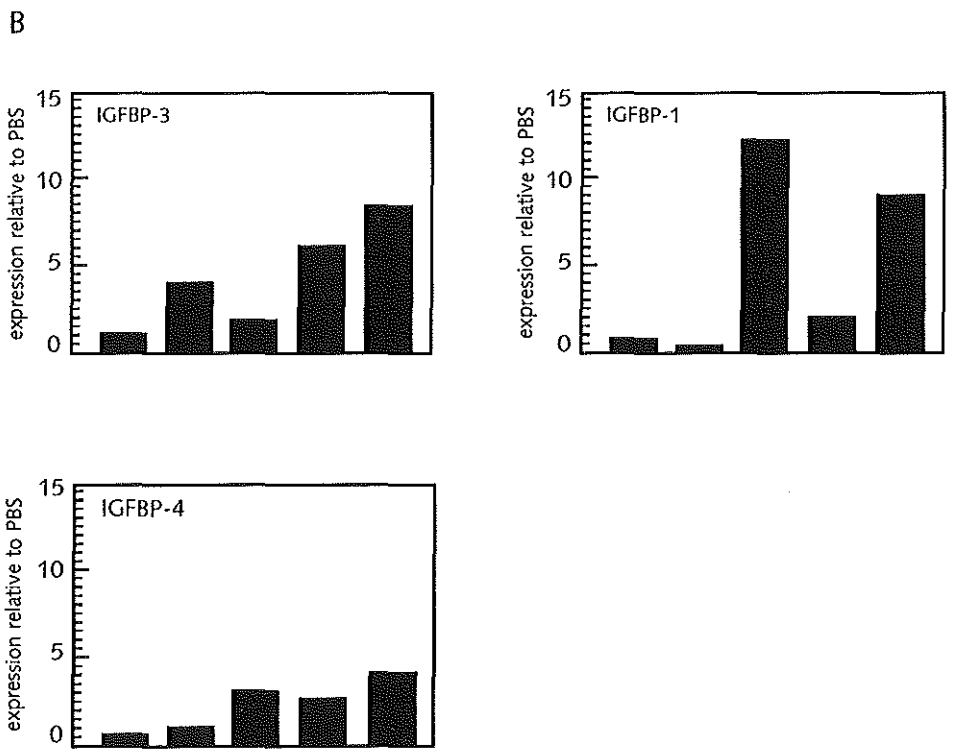


Figure 3

A. Western ligand blot of IGFBPs in pooled sera from dw/dw mice treated with hGH, IGFBP-1, IGF-I and IGF-I+IGFBP-1. SDS-PAGE of serum (2 (µl) and ligand blotting with ^{125}I-labeled-IGF-II was performed as described in Materials and Methods. B. shows the quantification of each band relative to the PBS control.

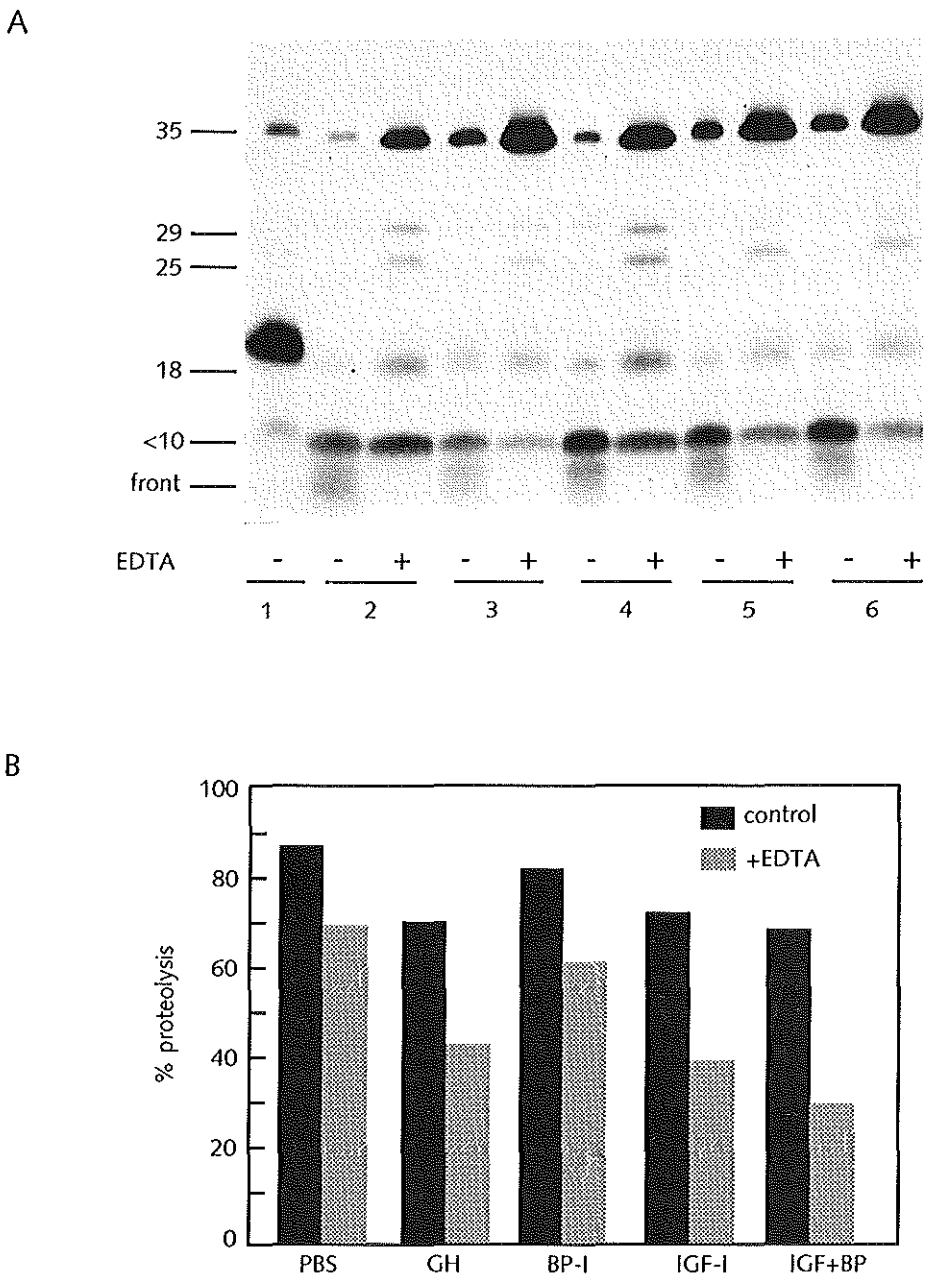


Figure 4
A. Proteolysis of IGFBP-3 by pooled sera from dw/dw mice. Non-glycosylated ^{125}I-labeled-IGFBP-3 was incubated with the various sera in the absence or presence of EDTA as described in Materials and Methods. Lane 1, human pregnancy serum (used as internal control of the protease assay); Lanes 2-6, dw/dw mice treated with: 2, PBS; 3, hGH; 4, IGFBP-1; 5, IGF-I; 6, IGF-I+IGFBP-1. B. Quantification of the amount of IGFBP-3 proteolyzed.

Discussion

Our data demonstrate that the growth promoting effect of IGF-I in Snell dwarf mice is inhibited when it is administered in a 1:1 molar ratio with IGFBP-1. The observed suppression is in accordance with data reported by Cox et al. after injection of IGFBP-1 in hypophysectomized rats (10). In transgenic mice overexpressing human IGFBP-1 (14,15,28) growth retardation is less than the observed growth inhibition in hypophysectomized rats or in dwarf mice after administration of IGFBP-1. This discrepancy can be due to the rather low IGFBP-1 serum concentrations measured in the transgenic animals (between 5 and 100 ng/ml). In the IGFBP-1 injected animals described in this study values of approximately 12,000 ng/ml were measured 2 hrs after the last injection. The latter concentration is in agreement with the high levels obtained in athymic mice after subcutaneous injection of comparable amounts of IGFBP-1 (29). Since recombinant hIGFBP-1 has a short half-life of approximately 2.5 hrs in mice (29), we administered IGFBP-1 three times a day. This scheme warrants a high level of IGFBP-1 during the day, which may explain the pronounced inhibition of IGF-I-induced growth in our dwarf mice. Increased serum IGFBP-1 levels of the same order of magnitude have also been measured in children suffering from chronic renal failure (CRF) (30,31). Since we show that IGFBP-1 administered at high concentrations can reduce IGF-I-stimulated growth *in vivo*, it is conceivable that the high IGFBP-1 concentrations in these children also play a role in their growth failure.

Regarding organ growth, we observed a differential pattern. Both GH and IGF-I are growth stimulatory for many organs in accordance with data reported before (22,32). Similar as for body length and body weight, we observed inhibition of IGF-I-induced growth by IGFBP-I in muscles and testes. IGFBP-1 alone had no effect on the growth of these organs. Kidneys, submandibular salivary glands and spleen, however, showed an enlargement after injection of IGFBP-1 alone, although for the latter two significance was not reached. Co-administration of IGFBP-1 and IGF-I results in additive effects on growth of these organs. The growth stimulation by the combination of IGF-I and IGFBP-1 is the highest in the kidneys.

The effects obtained by administration of IGFBP-1 are in marked contrast with those observed with IGFBP-3. IGFBP-1 is stimulatory for kidneys, spleen and submandibular salivary glands and inhibitory for growth of the thymus, whereas IGFBP-3 has no effect at all (27). With respect to body length and weight, both IGFBP-1 and IGFBP-3 have no effect when administered alone. Furthermore, they both neutralize IGF-I-induced growth. This contrasts with the observed IGF-I- induced growth stimulation of the muscles quadriceps femoris and the submandibular salivary glands, which is not affected by co-administration of either IGFBP-1 or IGFBP-3. In de kidneys, however, co-injection of IGFBP-3 diminished IGF-I-induced growth, whereas IGFBP-1 shows an additive effect together with IGF-I. In Fig.5 the relationship between kidney weight and body weight is plotted to illustrate these differences between treatment with IGFBP-1 and IGFBP-3 for the kidneys. These data demonstrate that *in vivo* IGFBP-1 may either inhibit or stimulate weight of organs in a tissue specific manner. Although IGFBP-3 shows similar actions, the organ specificity is different. Differences between IGFBP-1 and IGFBP-3 actions are also found in IGFBP-3 and IGFBP-1 transgenic mice. In IGFBP-3 transgenic mice total body growth is unaffected, selective organomegaly is demonstrated for the spleen, liver and heart. However, no effect is observed for the kidneys (33). In transgenic mice overexpressing IGFBP-1 under control of the metallothionein or phosphoglycerate

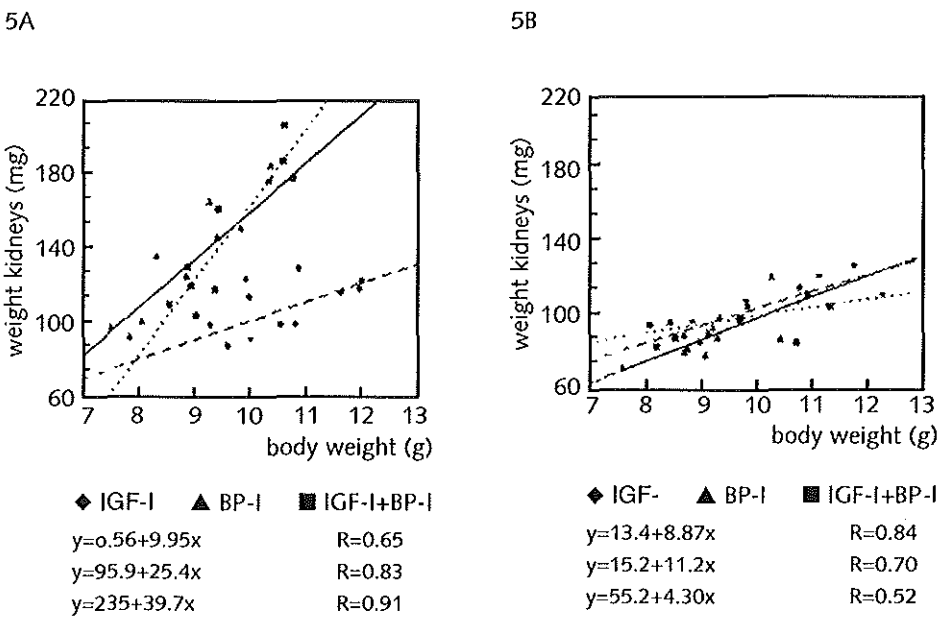


Figure 5
Kidney weight as a function of body weight after 4 weeks of treatment with IGF-I (◆), IGFBP-1 (▲) and IGF-I+IGFBP-1 (■) (left panel) and IGF-I, IGFBP-3 and IGF-I+IGFBP-3 (right panel). Covariance analysis shows statistical differences (P(0.05) between IGFBP-1 or IGF-I+IGFBP-1 treatment and the other treatments (PBS, IGF-I, IGFBP-3 and IGF-I+IGFBP-3).

promoter, growth is slightly affected and growth of the brain is markedly retarded, while weights of the kidneys are unaffected and splenomegaly is only observed in the phosphoglycerate IGFBP-1 transgenic mice (15,28).

In line with our results for the kidneys are those seen in potassium-depleted rats. While in these animals body weight gain was arrested, both absolute and relative weights of the kidney were significantly increased and this was further enhanced by IGF-I infusion. In addition, IGFBP-1 mRNA in the kidney was enhanced during fasting and protein restriction, suggesting, as in our study, a paracrine action of IGFBP-1 on the kidneys (34-36).

GH, IGFs and IGFBPs play an important role in the pathophysiology of the kidney (37,38). In experimental diabetes IGFBP-1, 2, 4 and 5 show marked changes in expression in the kidney, suggesting a possible involvement of these binding proteins in diabetic renal hypertrophy (38-41).

Differential effects of the treatments also become apparent in serum. GH and IGF-I increased serum IGFBP-3 as shown by us and others (22,42). High concentrations of IGFBP-1 do not influence IGF-I-induced serum IGFBP-3 concentrations. The slight decrease of IGFBP-3 protease as observed after treatment with IGF-I is similar to the effect of GH (26), and is not affected by co-injection of IGFBP-1. On the contrary, IGFBPs migrating at molecular weight of 24 kD and probably representing IGFBP-4, are upregulated both by GH, IGFBP-1 and IGF-I as suggested by their rise after treatment with these peptides.

IGF-I seems to accelerate the disappearance of IGFBP-1 from serum, resulting in inhibition of the IGF-I-induced hypoglycemia by the complex. IGFBP-1 alone does not cause hyperglycemia, confirming the results in hypophysectomized rats (10), but this contrasts with observations in normal rats (11) and IGFBP-1 transgenic mice (43) in which a rise in plasma glucose levels was observed. A possible explanation for this discrepancy can be that the normal glucocounterregulation with GH is lacking in GH-deficient animals.

The observed changes of IGF-I, IGFBPs and IGFBP protease in serum may be important in defining the local concentrations of these peptides and therefore contribute to tissue-specific growth regulation. The marked heterogeneity in the effects of IGFBP-1 on different organs is in line with the observations that tissues synthesize and secrete IGFBPs in a tissue-specific fashion (3,18,44). Therefore one can presume that the net effect of IGFs and IGFBPs in our dwarf mice differs per tissue, resulting in either enhancement or inhibition of IGF actions. These diverse actions of IGFBP-1 in our dwarf mice may complicate its use as a therapeutic agent as suggested for instance for the inhibition of tumor growth *in vitro* and *in vivo* (45).

Expression of IGFBPs during development in the rat and mouse kidney and the effects of diabetes and potassium depletion on their expression pattern have been studied extensively (36,38,39,46,47). Since the enlargement of kidneys as noticed in our mice was comparable to the enlargement of kidneys in the above mentioned disease states a detailed study concerning the mRNA and protein expression patterns of several constituents of the GH-IGF-axis related to morphological changes is desired. This will be the subject of the accompanying paper (Van Kleffens et al. 1999).

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5

Human IGFBP-1 inhibits IGF-1 stimulated body growth but stimulates growth of the kidney in Snell dwarf mice

Chapter 6

Effects of growth hormone, insulin-like growth factor-I and IGF-binding protein-1 administration on gene and protein expression of members of the IGF system in the kidney of Snell dwarf mice

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Abstract

The effects of administration of IGF binding protein-1 (IGFBP-1) alone and in combination with insulin-like growth factor-I (IGF-I) on kidneys of Snell dwarf mice were investigated in relation to administration of growth hormone (GH) and IGF-I. All treatments increased kidney length and weight significantly. In contrast, the effects on kidney morphology were different. Administration of IGFBP-1 or IGF-I + IGFBP-1 both caused dilatation of the thin limbs of Henle's Loop, while GH and IGF-I had no visible effect. GH, IGF-I + IGFBP-1 or IGFBP-1 caused a decreased mean number of nuclei per cortical area with a concomitant increased kidney weight compared to the control. In contrast, IGF-I administration resulted in an increase in nuclei number and kidney weight, indicating hyperplasia of the kidney.

In situ hybridization and immunohistochemistry showed specific changes of IGF system expression patterns in different structures of the kidneys of the treated animals. For mRNA, changes after IGF-I and IGF-I + IGFBP-1 administration were almost identical, while GH and IGFBP-1 administration gave different results. In contrast, regarding protein identical results were obtained after administration of IGFBP-1 and IGF-I + IGFBP-1, whereas GH and IGF-I treatment resulted in different changes.

In conclusion, when IGFBP-1 is administered with IGF-I, IGF-I effects on renal IGF system mRNA and protein expression are inhibited. Our results indicate that IGFBP-1 has an IGF-I independent, local effect on kidney morphology, proliferation and changes in mRNA and protein expression of the IGF system. IGFBP-1 seems to affect renal fluid exchange that may cause dilatations of the thin limbs of Henle's Loop.

Introduction

The growth promoting effects of the GH-IGF axis on body and organ growth seem to be highly dependent on the local distribution of the IGF-binding proteins (IGFBPs). In this respect, the role of IGFBP-1 in the physiology of the kidney is still poorly understood. In several disease states, it has been suggested that IGFBP-1 plays an important role. In man, circulating levels of IGFBP-1 are elevated at short term in diabetes and prolonged diabetes often results in kidney problems (Flyvbjerg, 1993; Jones and Clemmons, 1995; Fervenza et al., 1997). In experimental diabetes in rodents serum levels of IGFBP-1 are elevated as well, together with decreased IGF-I levels (Luo et al., 1992; Bach et al., 1994). In these animals, renal IGFBP-1 mRNA is increased in cortex and decreased in the medullary thick limbs of Henle's Loop, the site of IGFBP-1 expression in normal adult kidneys (Landau et al., 1995). IGF-I concentrations in the kidney are elevated in rats during the first three days of streptozotocin-induced diabetes. This initial increase seems to precede the earliest change in kidney function, such as the increase of glomerular filtration rate (Flyvbjerg et al, 1990). Interestingly, the increase in kidney IGF-I content is not caused by an increased mRNA expression, as renal IGF-I mRNA levels remain unchanged at short-term and decrease in long term diabetes (Flyvbjerg et al., 1990; Landau et al., 1995). Therefore, it is tempting to speculate that IGF-I is trapped by IGFBPs, such as IGFBP-1 (Philip et al., 1994). The elevated renal IGFBP-1 expression at the onset of diabetes supports this idea (Landau et al., 1995).

Other kidney diseases as well seem to involve IGFBP-1 action. Patients with chronic renal failure (CRF) have high IGFBP-1 serum levels, that correlate inversely with glomerular filtration rate (Lee et al., 1989; Hokken-Koelega et al., 1991; Tönshoff et al., 1995; Powell et al., 1996). However, serum GH and IGF-I levels are normal or elevated, while serum IGF bioactivity is low (Hokken-Koelega et al., 1991, 1994; Lee et al., 1993; Flyvbjerg, 1994; Tönshoff et al., 1995). In CRF serum IGFBP-1 levels have been correlated directly with serum IGF-binding activity (Lee et al., 1989), which suggests an inhibiting role for IGFBP-1 on serum IGF bioavailability. Interaction of IGF-I and IGFBP-1 was also suggested by Chin and colleagues (1992). From their experiments with hypophysectomized rats they concluded that IGF-I in the medullary thick limbs of Henle's Loop acts locally, being modulated by IGFBP-1.

Dietary potassium depletion, another kidney model, reduces body weight gain and organ growth, except for kidney which increases in wet weight as a result of renal hypertrophy (Gustafson et al., 1973; Flyvbjerg et al., 1991). Potassium-deficient rats showed increased IGFBP-1 renal mRNA and immunohistochemistry revealed hyperplasia of distal nephron segments and increased amounts of IGFBP-1 protein in kidney of these rats (Rohan et al., 1997). This increase in renal IGFBP-1 mRNA expression in potassium-deficient rats is not affected by IGF-I infusion (van Neck et al., 1997).

In Chapter 5 we showed that treatment of homozygous Snell dwarf mice with hGH and IGF-I, for a period of 4 weeks, caused an increase in body length and body weight. IGFBP-1, however, inhibited IGF-I induced total body growth (van Buul-Offers et al., Chapter 5). We also showed that treatment with hGH and IGF-I had a pronounced effect on kidney size. In contrast to the reduction of total body growth, IGFBP-1 had a stimulatory effect on the kidney. The aim of the present study was to investigate the effects of IGFBP-1 on the kidneys of Pit-1 deficient Snell dwarf mice in further detail. Therefore, the effects of treatment with IGF-I, IGFBP-1 and IGF-I +IGFBP-1 on kidney

morphology, kidney specific mRNA and protein expression of the different members of the IGF system in Snell dwarf mice were analyzed. In an effort to discriminate between direct IGF-I/IGFBP-1 effects and GH dependent effects, we also administered hGH.

Materials and Methods

Materials

The experimental design has been described by S.C van Buul-Offers et al. (Chapter 5). Briefly, dwarf mice (n=5 per group) were treated by subcutaneous injection with PBS, hGH (16.6 mU/day); IGF-I (30mg/day); hIGFBP-1 (105mg/day) and equimolar amounts of IGF-I + IGFBP-1 (30+105mg/day), during 4 weeks.

Tissue isolation

Animals were killed 2 hrs. after the last injection. Kidneys were removed and weighed to the nearest mg. One kidney was frozen in liquid nitrogen for Northern blot analysis (see below); the other was immediately fixed overnight in 4% paraformaldehyde, and embedded in paraffin. 4µm sections were cut and mounted on aminopropyl trioxysilane-coated slides. Slides were dried at 37°C for 3 days. In order to obtain optimal comparison between the different treatments, all organs have been fixed, embedded in paraffin, and mounted on slides similarly and at the same time. Kidney sections of all groups have been mounted on the same slide. As a result the experimental parameters of *in situ* hybridization and immunohistochemistry experiments per probe or antibody, respectively, were the same for all groups.

Morphology

To determine kidney size, kidneys were cut sagitally into two equal parts before embedding in paraffin, followed by sectioning. The central sections of kidneys of all treatment groups (PBS group as a control) were measured with a ruler. To count the number of nuclei per square, sections were placed under a light microscope (Zeiss) with a grid. Per section 5 cortical and medullar areas were counted in triplicate of 5 animals per treatment group (areas of 0.25 mm^2; cortex: tubular nuclei in the outer cortical areas were counted. These areas did not contain glomeruli or medullary rays). Assignment of the various nephron parts was done as described by Lindenbergh-Kortleve et al. (1997). Hence, thick limbs of Henle's Loop comprise proximal and distal straight tubules. Proximal and distal convoluting tubules are called proximal and distal tubules, respectively.

Northern blot analysis of mRNA expression of members of the IGF system

Total RNA of nitrogen frozen kidney was isolated by the guanidinium thiocyanate method as described (Chomczynski and Sacchi, 1987). Glyoxal-denatured RNA was electrophoresed in 1% agarose gels submerged in 10 mM sodium phosphate pH 7.2 and transferred to nylon membranes (Hybond N^+, Amersham, 's Hertogenbosch, The Netherlands). Hybridization of filters was performed overnight with 1-2x10^6 cpm per ml of ^{32}P-labeled cDNA fragments of mouse IGF-I, IGFBP-1, -2, -3, -4, -5 and -6 (Schuller et al, 1994) at 65°C (Church and Gilbert, 1984). Quantification of Northern blots was

performed by scanning on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA), using ImageQuant software. All measured results were expressed relative to 18S rRNA levels as mean ±SEM.

Probe preparation (in situ hybridization)

Specific mouse IGFBP cRNA probes were transcribed from IGFBP-2, -3, -4, -5, -6 cDNAs as described by Schuller et al. (1994). The mouse cDNA *Sph*I-*Sac*I fragment was used as template for the IGFBP-1 cRNA probe and was cloned into pTZ18R or pTZ19R (Pharmacia, Uppsala, Sweden) for the antisense and sense probes, respectively. Similarly, mouse IGF-I and -II (cDNAs kindly provided by Dr GI Bell, Howard Hughes Institute, Chicago, IL, USA) fragments were subcloned into pTZ18 and pTZ19 (*Eco*RI for IGF-I and *Bam*HI/*Sac*I for IGF-II). A 265 bp *Eco*RI/*Sma*I fragment of the rat type I IGF receptor cDNA ligated in PGEM3 (Promega, Madison,WI) was kindly provided by Dr H Werner and Dr D LeRoith (National Institutes of Health, Bethesda, MD) and was used as template for the type I IGF receptor probe. Digoxigenin-11-UTP labeled RNA probes were prepared according to the manufacturer's prescription (Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany), using T7 or SP6 RNA polymerase.

In situ hybridization

A non-radioactive ιν σιτυ hybridization was performed, as described before (Lindenbergh-Kortleve et al., 1997). Hybridization was performed overnight at 55°C in a humid chamber in a hybridization solution containing 50% deionized formamide, 10% dextran sulfate, 2xSSC, 1x Denhardt's solution, 1µg/ml tRNA, 250 µg/ml herring sperm DNA and the respective probes at a concentration of 100 ng/ml. After various washes (50% formamide/2xSSC, 50% formamide/1xSSC and 0.1xSSC) sections were incubated with RNase T1 (2U/ml in 1mM EDTA/2xSSC) for 15 minutes at 37°C. The digoxigenin labeled hybrids were detected by antibody incubation performed according to the manufacturer's recommendations (Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany). PVA (Polyvinylalcohol, MW 31-50 kD, Aldrich Chemical Co. Milwaukee, WIS, USA) enhanced staining with BCIP (5-bromo-4-chloro-3-indolyl-phosphate), NBT (4-nitroblue tetrazolium chloride) and levamisol was stopped when the desired intensity of the blue precipitate was reached. This was dependent on the relative abundancy of the respective mRNAs. Sections were counterstained with Nuclear red solution and mounted with Euparal (ChromaGesellschaft, Stuttgart, Germany). Control sections for morphological analysis were either stained with PAS, or with hematoxylin and eosin. *In situ* hybridized sections were analyzed blindly by two independent investigators, in at least five separate observations. Negative controls for *in situ* hybridization were performed using sense probes, which never gave any significant staining.

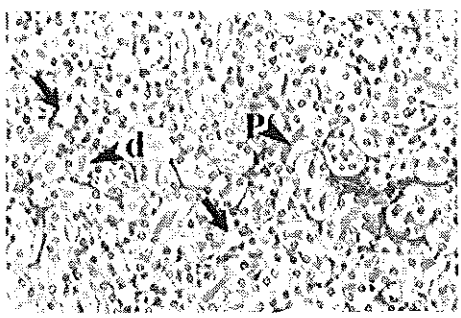
Immunohistochemistry

Polyclonal antibodies to mIGFBP-1, -2, -3, -4, -5 and -6 were generated as described (van Kleffens et al., submitted). Briefly, synthetic peptides (ID-DLO, Lelystad, the Netherlands), corresponding to parts of the variable region of the various mouse IGFBPs (IGFBP-1 and IGFBP-2: amino acid position (aa)174-189; IGFBP-3: aa200-213; IGFBP-4: aa155-170; IGFBP5: aa176-191; IGFBP-6: aa128-143), were coupled to the carrier keyhole limpet hemocyanin (KLH; Pierce, Rockford, Illinois, USA) and injected into rabbits.

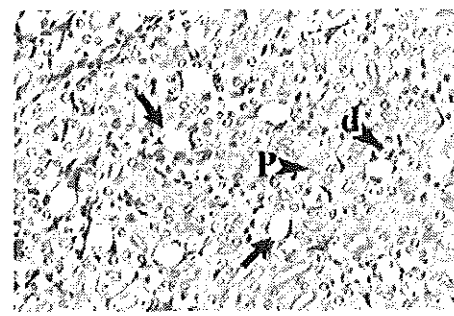
The resulting antisera were used in an immunohistochemical staining, making use of the peroxidase-anti-peroxidase (PAP) method. The anti mIGFBP-1 and -3 polyclonals were diluted 1:150, while the anti mIGFBP-2, -4, -5 and -6 polyclonals were used in a dilution of 1:250. Staining patterns were analyzed in comparison to their respective pre-immune sera, diluted 1:10. Unlabeled goat anti-rabbit immunoglobulins (Dako, Glostrup, Denmark; 1:50) were used as second antibody and rabbit PAP (Dako, Glostrup, Denmark; 1:100) as a linker. Staining was performed with diaminobenzidine (DAB; Fluka Chemika, Buchs, Switzerland; 0.75 mg/ml). Nuclei were made visible with Mayer's haematoxilin.

Statistical analysis

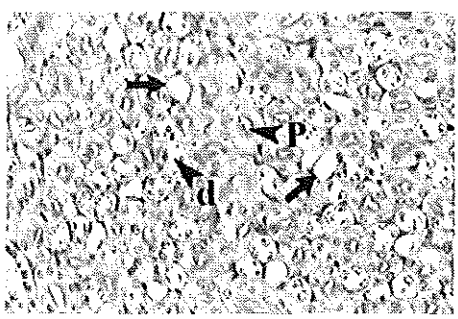
The differences in kidney length, kidney weight and mean number of nuclei per area after administration were scored for significance using the Student's *t*-test or a Scheffé *F* test (Scheffé, 1979).



1A



1B



1C

Figure 1
Morphology of the renal outer medulla of Snell dwarf mice after different treatments.
A: Control. B: IGFBP-1 treatment. C: IGF-1 + IGFBP-1 treatment.
Abbreviations: P=proximal tubules, d=distal tubules. Arrows: dilatations of the thin limbs of Henle's Loop.

Results

Morphology

To investigate possible differences in phenotype between kidneys of untreated, phenotypically normal heterozygous (+/dw) and untreated homozygous Snell dwarf mice, PAS stained sections were examined. No differences in morphology could be detected. Although kidneys of homozygous Snell dwarf mice were smaller, they were proportional to their decreased body size. Figure 1A shows the morphology of the outer medulla of the dwarf mouse kidney. After treatment with IGFBP-1 (Fig. 1B) and IGF-I +IGFBP-1 (Fig. 1C) kidneys of Snell dwarf mice demonstrated pronounced dilatation of thin limbs of Henle's Loop in the medulla. This dilatation could not be observed in IGF-I and hGH treated mice. Regardless of the treatment kidney weight increased simultaneously with kidney size. To determine if increased kidney weight reflected an increase in cell number, nuclei were counted in cortical and medullar areas. Cortical and medullar count results were similar (medullar data not shown). Kidney length and kidney weight were plotted against the number of cell nuclei per cortical area (Fig. 2). In glomeruli no changes in numbers of nuclei between the different treatment regimen were observed (data not shown). In the tubular cortical region and compared to control, however, a decreased number of nuclei per area was found for all treatments, except for IGF-I. After treatment with IGF-I, kidney length and weight were increased, while the cell number per area did not change. In contrast, treatment of Snell dwarf mice with hGH, IGFBP-1 or IGF-I + IGFBP-1 caused an increase in kidney length and weight, while the cell number per cortical area decreased simultaneously.

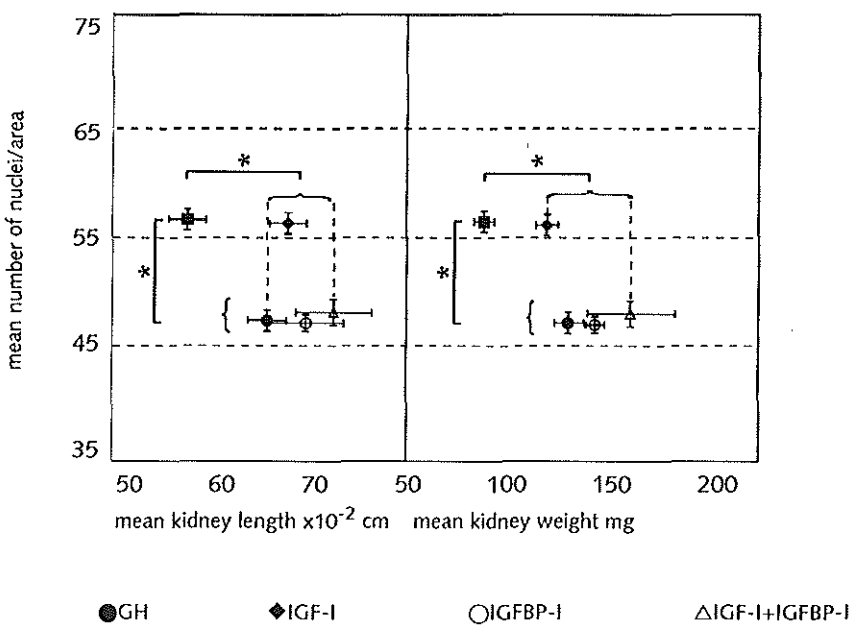


Figure 2
*Mean kidney length (per kidney) and weight (per pair of kidneys) ±SEM were plotted against the mean number of nuclei ±SEM per cortical area, for all treatments. *:significant difference with p=0.005.*

Table 1

Changes of mRNA expression of members of the IGF system in the kidney of dwarf mice after various treatments

Structure	Cortex	glomeruli -mesangial cells	glomeruli -Bowman's capsule	peritubular capillaries	distal/collecting tubules	proximal tubules	thick limbs of Henle's Loop	Medulla	thick limbs of Henle's Loop	thin limbs of Henle's Loop	peritubular capillaries
mRNA localization											
Control		BP4	IGF-I	IGF-I	IGF-I	IGF-II	IGF-I		IGF-I	BP2	IGF-I
		BP5	IGF-II	BP3	IGF-II	tIr	IGF-II		IGF-II		BP3
			tIr	BP5	tIr	BP2	tIr		tIr		BP4
			BP2		BP2	BP4	BP2				BP5
					BP5		BP4				
Changes in mRNA, compared to control											
GH					BP2▲	IGF-I+	BP2▲				IGF-I▲
											BP5▼
IGF-I					BP2▲	tIr▼	IGF-I▼		IGF-I▼		
							tIr▼				
							BP2▲				
IGFBP-I					IGF-I▲	IGF-I+	IGF-I▲		IGF-I▲	BP2▲	
					BP2▲	IGF-II▲	IGF-I▲				
					BP5▲	tIr▲	tIrv				
						BP2▲	BP2▲				
IGF-I+IGFBP-I					BP2▲	tIr▲	IGF-I▼		IGF-I▼		
							tIr▲				
							BP2▲				

Data are determined compared to the control
▲ *increased expression*
▼ *decreased expression*
\+ *induced expression*
BP:IGFBP
tIrec *type I IGF receptor*

mRNA expression of members of the IGF system

The IGF system mRNA expression patterns in the kidneys of Snell dwarf mice were similar to those described previously for kidneys obtained from normal Balb/c mice (Lindenbergh-Kortleve et al., 1997). Table 1 shows the expression patterns of the members of the IGF system of the control dwarfs. For the GH, IGF-I, IGFBP-1 and IGF-I + IGFBP-1 treatment groups differences compared to controls are given. Marked differences between the controls and IGFBP-1 treated mice are illustrated in Figures 3 and 4. Expression of mIGFBP-1 and mIGFBP-6 mRNA in kidney of all treatment groups was limited and no differences were shown between the various treatment modalities (data not shown).

Control

In kidneys of Snell heterozygous and homozygous dwarf mice, IGF-I mRNA was located in Bowman's capsule, peritubular capillaries of the inner cortex and the medulla, distal/collecting tubules and thick limbs of Henle's Loop (Fig. 3A). Localization of IGF-II mRNA was found in Bowman's capsule, proximal and distal/collecting tubules and thick limbs of Henle's Loop (Fig. 3B). Like IGF-II, type I IGF receptor mRNA was localized in Bowman's capsule, proximal and distal/collecting tubules and thick limbs of Henle's Loop (Fig. 3C). IGFBP-2 mRNA was found in Bowman's capsule, thick (inner cortex) and thin limbs of Henle's Loop, distal/collecting tubules and to a lesser extent in the proximal tubules (Fig. 3D). Localization of IGFBP-3 mRNA was confined to peritubular capillaries of the outer cortex and the medulla. Signal of IGFBP-4 mRNA was found in mesangial cells of glomeruli, peritubular capillaries of the outer medulla, proximal tubules and cortical thick limbs of Henle's Loop. IGFBP-5 mRNA was localized in mesangial cells of glomeruli, juxtaglomerular cells, peritubular capillaries of the outer cortex and of the medulla, and distal/collecting tubules.

GH treatment

In GH treated dwarf mice renal IGF-I mRNA expression was induced in the proximal tubules and was increased in peritubular capillaries of the medulla. Staining of IGFBP-2 mRNA was stronger in distal/collecting tubules and thick limbs of Henle's Loop (cortex). In contrast, IGFBP-5 mRNA signal was diminished in peritubular capillaries of the medulla (Table 1).

IGF-I treatment

The kidneys of IGF-I treated dwarf mice demonstrated slight differences when compared to the control group. IGF-I mRNA expression was decreased in both cortical and medullary thick limbs of Henle's Loop. A diminished signal of type I IGF receptor mRNA expression in cortical thick limbs of Henle's Loop and in proximal tubules was seen. Furthermore, IGFBP-2 mRNA expression was more pronounced in distal/collecting tubules and cortical thick limbs of Henle's Loop (Table 1).

IGFBP-1 treatment

IGFBP-1 administration caused the most significant changes in renal mRNA expression. IGF-I mRNA expression was increased in distal/collecting tubules and was induced in proximal tubules. Furthermore, IGF-I mRNA signal was increased in thick limbs of Henle's

Loop (cortex and medulla) (Fig. 4A). Similarly, staining of IGF-II mRNA was enhanced in cortical thick limbs of Henle's Loop (Fig. 4B). Signal of type I IGF receptor mRNA was increased in cortical thick limbs of Henle's Loop (Fig. 4C) and proximal tubules. Increased IGFBP-2 mRNA signal was also found in proximal and distal tubules of the cortex and thin limbs of Henle's Loop (Fig. 4D). Furthermore, elevated signal was found for IGFBP-5 in distal tubules. More cells per tubule demonstrated IGFBP-5 mRNA expression after IGFBP-1 treatment.

IGF-I + IGFBP-1 treatment

Combined administration of IGF-I + IGFBP-1 showed the same changes of mRNA patterns as found for the IGF-I treated group, with one exception: type I IGF receptor mRNA signal was stronger in proximal tubules and thick limbs of Henle's Loop. The latter resembles the pattern observed in IGFBP-1 treated mice (Table 1).

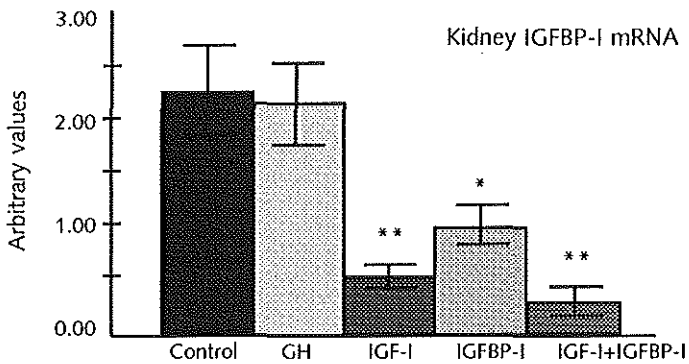


Figure 5

*Relative Northern blot results of renal IGFBP-1 mRNA expression after treatment of Snell dwarf mice with PBS (control), GH, IGF-I, IGFBP-1 and IGF-I + IGFBP-1 for 4 weeks. Values are based on densiometric measurements of Northern blots, compensated for RNA loading differences and expressed in arbitrary units as means ± SEM (n=5). *P=0.01, **P<0.001, statistical significance level between the indicated group and control.*

Northern-blot analysis of IGF system expression

Samples of kidney mRNA of all treatment groups were probed with different IGF system components. Only IGFBP-1 renal mRNA expression changed significantly upon treatment with IGF-I, IGFBP-1 and IGF-I + IGFBP-1. Renal IGFBP-1 expression was significantly decreased by IGF-I (3-fold, $P<0.001$), IGFBP-1 (2-fold, $P=0.01$) and IGF-I + IGFBP-1 (7-fold, $P<0.001$) treatment (Fig. 5). IGFBP-2 and -6 mRNA expression could not be detected.

Protein localization

Results of immunohistochemistry of kidneys of the different groups are summarized in Table 2. Marked differences between controls and IGFBP-1 treated mice are given in Figures 6 and 7. As for mRNA expression, IGFBP-1 and -6 proteins could not be detected immunohisto-chemically.

Table 2

Changes in protein localization of members of the IGF system in the kidney of dwarf mice after various treatments

Structure	Cortex	glomeruli -mesangial cells	glomeruli -Bowman's capsule	peritubular capillaries	distal/collecting tubules	proximal tubules	thick limbs of Henle's Loop	Medulla	thick limbs of Henle's Loop	thin limbs of Henle's Loop	peritubular capillaries
protein localization											
Control		BP4	IGF-I	BP3	IGF-I	IGF-II	tIr		BP4	BP2	BP3
					IGF-II	tIr	BP2			BP4	
					tIr	BP2	BP4				
					BP3	BP3					
					BP4	BP4					
						BP5					
Changes in protein, compared to control											
GH					tIr▼	tIr▲	tIr▲		BP4▲		
						BP5▲					
IGF-I						BP5▼					
IGFBP-I				BP3▼	tIr▼	IGF-I+	tIr▼		BP4▲		BP3▼
					BP3▲	IGF-II▲	BP2▲				
						tIr▼					
						BP2▼					
						BP3▲					
						BP4▲					
						BP5▲					
IGF-I+IGFBP-I				BP3▼	tIr▼	IGF-I+	tIr▼		BP4▲		BP3▼
						tIr▼	BP2▼				
						BP2▼					
						BP3▲					
						BP4▲					
						BP5▲					

Data are determined compared to the control
▲ *increased expression*
▼ *decreased expression*
\+ *induced expression*
BP:IGFBP
tIrec *type I IGF receptor*

Control

The kidneys of control dwarf mice revealed weak staining for IGF-I in Bowman's capsule and distal/collecting tubules. IGF-II was also found in distal/collecting tubules and, in addition, in the proximal tubules. Type I IGF receptor protein was localized in thick limbs of Henle's Loop, dispersed in distal/collecting tubules and weakly in proximal tubules (Fig. 6A). IGFBP-2 protein was detected in proximal tubules and thick and thin limbs of Henle's Loop (Fig. 6B). IGFBP-3 protein was localized in peritubular capillaries of the outer medulla (Fig. 6C) and in the cortex in peritubular capillaries nearby glomeruli. Moreover, staining was found in proximal tubules and in some cells of distal/collecting tubules. IGFBP-4 localization was shown in thick limbs of Henle's Loop, proximal convoluting tubules, peritubular capillaries of glomeruli and little in thin limbs of Henle's Loop (Fig. 6D). IGFBP-5 staining was weak and confined to proximal tubules.

GH treatment

When comparing the GH infused group with the control group, type I IGF receptor staining was more intense in proximal tubules and cortical thick limbs of Henle's Loop. Moreover, staining in distal/collecting tubules was decreased and was less than in proximal tubules. IGFBP-4 staining was elevated in thick limbs of Henle's Loop. The staining of IGFBP-5 protein was increased in proximal tubules of GH treated animals. All other members of the IGF system showed identical patterns as in kidneys of control animals (Table 2).

IGF-I treatment

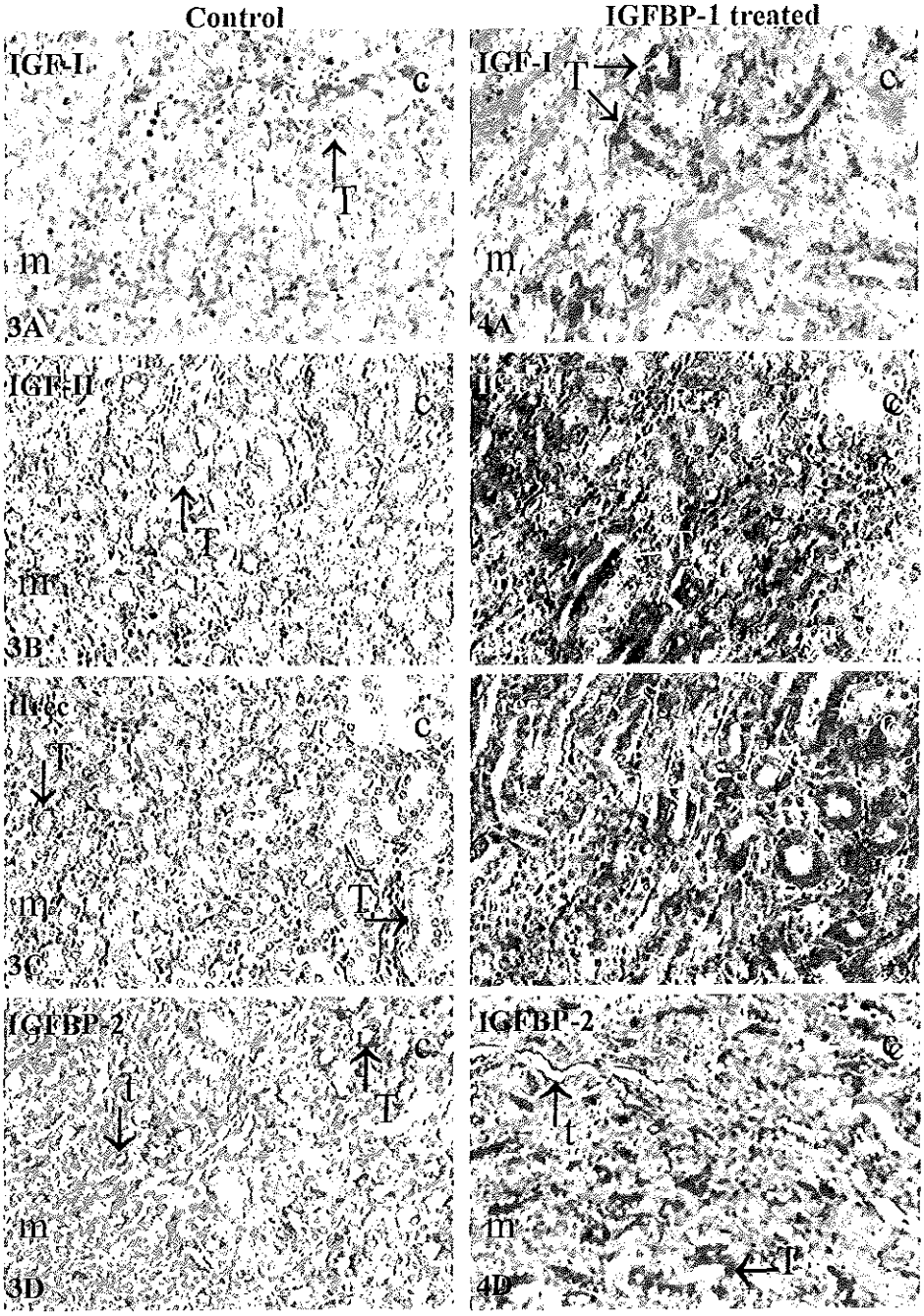
Type I IGF receptor protein was localized both in distal and proximal tubules, at the same, weak, level. Staining of thick limbs of Henle's loop is unchanged. Furthermore, staining for IGFBP-5 protein in proximal tubules was diminished. All other expression patterns were comparable with the controls.

IGFBP-1 treatment

IGF-I was induced by IGFBP-1 treatment. Protein could be detected in a patchy pattern in the brush borders of proximal tubules. IGF-II staining was increased in proximal tubules. Type I IGF receptor protein staining was decreased in proximal and distal/collecting tubules and cortical thick limbs of Henle's Loop. However, staining of distal/collecting tubules was more intense than staining of proximal tubules (Fig. 7A). Compared to the control group, IGFBP-2 signal was diminished in proximal tubules and thick limbs of Henle's Loop (Fig. 7B). IGFBP-3 staining showed a moderate increase in distal and proximal tubules. However, it was decreased in peritubular capillaries in both cortex and medulla (Fig. 7C). IGFBP-4 staining was more pronounced in proximal tubules and thick limbs of Henle's Loop (outer medulla)(Fig. 7D). IGFBP-5 showed a more intense staining in proximal tubules, with respect to the control group.

IGF-I + IGFBP-1 treatment

The staining patterns of the IGF system components after combined IGF-I + IGFBP-1 treatment are identical to the patterns found upon IGFBP-1 treatment. However, there was one exception for the IGF-II staining, that resembled the control group pattern.


Control
IGFBP-1 treated
IGF-I
IGF-I
IGF-II
IGF-II
IGFBP-2
IGFBP-2
3A
4A
3B
3C
3D
4D

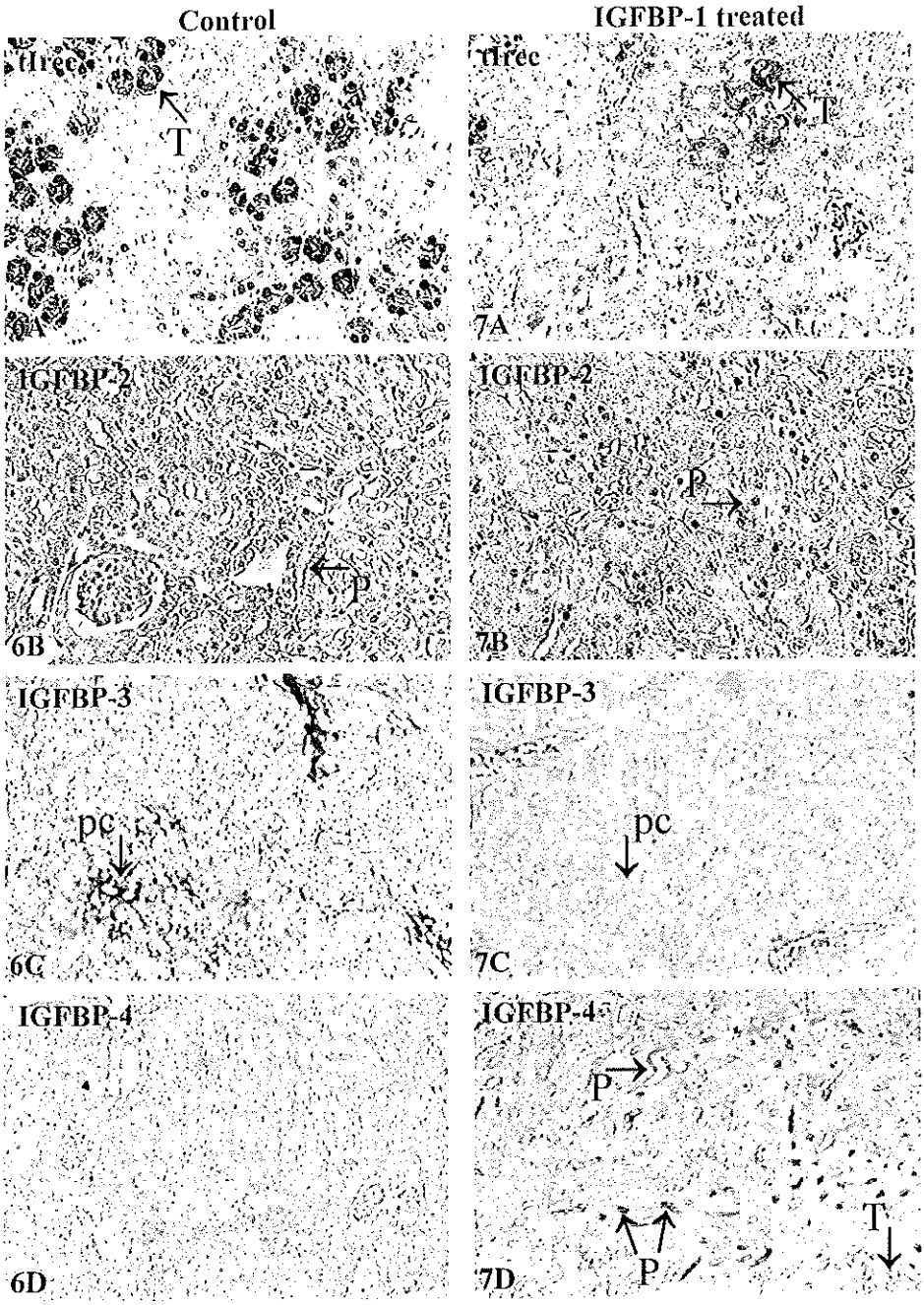

Control
IGFBP-1 treated
Ttrec
T
6A
7A
IGFBP-2
P
6B
7B
IGFBP-3
pc
6C
7C
IGFBP-4
6D
7D

Figure 3
Non-radioactive in situ mRNA hybridization with DIG-labeled cRNA probes of kidneys of control dwarf mouse. A: IGF-I; B: IGF-II; C: type I IGF receptor; D: IGFBP-2. Abbreviations: T=thick limbs of Henle's Loop; t= thin limbs of Henle's Loop; m=medullary side; c=cortical side.

Figure 4
Non-radioactive in situ mRNA hybridization with DIG-labeled cRNA probes of kidneys after IGFBP-1 treatment. A: IGF-I; B: IGF-II; C: type I IGF receptor; D: IGFBP-2. Abbreviations: T=thick limbs of Henle's Loop; t= thin limbs of Henle's Loop; m=medullary side; c=cortical

Figure 6
Immunohistochemistry with DAB staining, of cortical sections of kidneys of control dwarf mice. A: type I IGF receptor; B: IGFBP-2; C: IGFBP-3; D: IGFBP-4. Abbreviations: pc=peritubular capillaries, T=thick limbs of Henle's Loop, P=proximal tubules.

Figure 7
Immunohistochemistry with DAB staining, on kidney cortical sections after IGFBP-1 administration to Snell dwarf mice. A: type I IGF receptor; B: IGFBP-2; C: IGFBP-3; D: IGFBP-4. Abbreviations: pc=peritubular capillaries, T=thick limbs of Henle's Loop, P=proximal tubules.

Discussion

Morphological effects

Human IGFBP-1 inhibits IGF-I stimulated body growth, but stimulates growth of the kidney in Snell dwarf mice (van Buul-Offers et al., Chapter 5). In the present study the effects of GH, IGF-I and IGFBP-1 administration on the morphology and the expression pattern of members of the IGF-system in kidneys of Snell dwarf mice were analysed. Treatment with IGFBP-1 alone or in combination with IGF-I resulted in an extensive change in kidney morphology and pattern of IGF system expression. An increased wet weight of the kidney was observed, likely caused by fluid filled dilatations of thin limbs of Henle's Loop. This increased kidney weight occurred with a decreased mean number of nuclei per (outer) cortical area. Thus, we may conclude that short-term IGFBP-1 or IGF-I + IGFBP-1 administration affected renal intracellular fluid accumulation and not cellular proliferation. This is in contrast with administration of IGF-I alone resulting in enlarged kidney size, due to enhanced kidney cell proliferation. The enlarged kidneys after GH treatment are the result of fluid accumulation, as indicated by the increased kidney length or weight with a simultaneous diminished number of nuclei per cortical area.

This study is comparable with a previous study by van Buul-Offers and coworkers, where the effects of IGF-I and IGFBP-3 on total body and organ growth were investigated (van Buul-Offers et al., 1995). In contrast to IGFBP-1, IGFBP-3 inhibits IGF-I induced kidney growth. In addition, administration of IGFBP-3 alone does not affect kidney size indicating differential roles for IGFBP-1 and IGFBP-3 in kidney.

The results of our present study seem to be in contrast with the observations in transgenic mouse models overexpressing IGFBP-1, where no gross changes in kidney size were described (D'Ercole et al., 1995). This discrepancy likely can be explained by the fact that the promoters and the expression patterns of the different transgenes never resulted in elevated IGFBP-1 serum levels.

The enlarged kidneys resulting from administration of GH or IGF-I to the Snell dwarf mice in the present study is in confirmation with a previous report (Hirschberg, 1993). Overexpression of GH and/or IGF-I in transgenic mice affects renal morphology, however, the effects seen were different from those obtained in our study (Quaife et al., 1989; Wanke et al., 1991). Kidneys of GH transgenic mice showed among others glomerulosclerosis and tubular cysts (Brem et al., 1989). The effect of supranormal levels of IGF-I on kidney was milder, resulting in enlarged glomeruli (Quaife et al., 1989). The circulating IGF-I levels of IGF-I transgenic mice and of the IGF-I treated dwarf mice in our study were similar (540 and 417.2 ng/ml, respectively) (Mathews et al., 1988), but the effects on kidney morphology were quite different. In serum of untreated Snell dwarf mice, IGF-I levels are low and IGF-I will not accumulate in the kidney. We found no elevation of IGF-I mRNA in kidney tissue after administration of IGF-I or IGF-I + IGFBP-1. However, IGF-I mRNA levels in kidney tissue of IGF-I trangenic mice were increased 2.7 fold (Doi et al., 1990). This may explain why no morphological changes occurred after administration of IGF-I.

In addition, in the streptozotocin (STZ) diabetes rat model renal hypertrophy is preceded by elevated renal IGF-I levels, while renal IGF-I mRNA is not changed (Bach, 1992; Flyvbjerg et al., 1990). After 4 days IGF-I levels return to normal values, which suggests a role for IGF-I in the initial rapid growth phase and not during maintenance of the diseased state (Flyvbjerg et al., 1988). In GH deficient rats STZ diabetes inhibits renal hypertrophy. Moreover, renal IGF-I levels are less elevated, indicating GH dependency of IGF-I

associated renal hypertrophy (Flyvbjerg et al., 1988; Flyvbjerg et al., 1992; Muchaneta-Kubura et al., 1994; Jacobs et al., 1997).

Administration of IGF-I in combination with IGFBP-1 did not give additional morphological effects, in comparison with administration of IGFBP-1 alone. Hence, we hypothesize that IGFBP-1 has an effect on kidney physiology, independent of circulating levels of IGF-I and that pre-incubation of IGFBP-1 with IGF-I will not block this activity of IGFBP-1. A similar IGF independent action for IGFBP-1 has been suggested already in a streptozotocin induced diabetes model (Feld and Hirschberg, 1996). Feld and Hirschberg suggested that IGFBP-1 may have a stimulating effect on kidney growth through mitogenic actions via $\alpha_5\beta_2$-integrins. Another indication for IGFBP-1 action on kidney is given by experiments with hypokalemic rats (Rohan et al., 1997; van Neck et al., 1997). The most significant finding is the increase of renal IGFBP-1 mRNA in these rats. Moreover, increased immunoreactive IGFBP-1 is spatially associated with tubular hyperplasia. These results suggest that IGFBP-1 may play a major role in distal nephron adaptation in potassium depleted rats.

As in our study hyperplasia, resulting from IGF-I administration, did not occur after administration of IGF-I + IGFBP-1, we conclude that IGFBP-1 inhibits the proliferative effects of IGF-I on kidney cell growth. This is in confirmation with the suggestion that IGFs are trapped by IGFBP-1, in experimental diabetes and in CRF patients (Hokken-Koelega et al., 1991, 1994; Lee et al., 1993; Flyvbjerg et al., 1994; Philip et al., 1994; Tönshoff et al., 1995).

The finding that no effect on kidney morphology was seen in our study upon treatment with GH, can be explained by the fact that circulating levels of GH in transgenic mice were far higher than can be expected after administration of GH in our (dwarf) model (3-900 ng/ml) (Brem et al., 1989).

Effects on renal expression of IGF sytem components

The effect of IGFBP-1 treatment on expression patterns of IGF system components mRNA and protein was most prominent. Furthermore, the administered compounds had different effects on mRNA expression and protein localization. Most remarkable was the finding that effects of administration of IGFBP-1 alone on IGF system protein levels were similar to the effects of IGFBP-1 administration in combination with IGF-I. Together with the finding that the morphological changes after IGFBP-1 alone or IGF-I + IGFBP-1 were identical, this indicates that IGFBP-1 plays an important role in kidney morphology.

In contrast, the effects on IGF system mRNA levels of IGF-I were almost identical to those of IGF-I + IGFBP-1. Thus, the effects of IGFBP-1 on renal IGF system mRNAs seemed to be blocked by IGF-I. These results indicate that serum IGF-I affected renal mRNA expression of IGF system components, albeit limited. Summarized, the changes on mRNA expression by IGF-I were not influenced by combined administration of IGF-I with IGFBP-1, whereas both treatments resulted in different kidney morphology. Therefore, we conclude that the changes in renal mRNAs by IGF-I did not cause the IGF-I induced growth.

As the IGFs and IGFBPs are secreted proteins, mRNA levels and localization may deviate from protein levels and localization. Our results show that there was a considerable overlap in localization of mRNA and protein. As compared with protein, a larger number of different members of the IGF system was detected at the mRNA level. However, it appeared that mRNA expression in a given cell type not necessarily leads to the detectable presence of the corresponding protein in the same cell type, as the IGFBPs are secreted proteins.

Remarkably, in the thin limbs of Henle's Loop, that became dilatated after treatment with IGFBP-1 alone or in combination with IGF-I, only IGFBP-2 mRNA expression was detectable. This IGFBP-2 mRNA expression was more pronounced only after IGFBP-1 treatment, which was not reflected in changes in protein detection.

Discrepancies between mRNA and protein of IGF system components in the kidney have been reported previously in several studies. For example, the majority of IGF-I protein in kidney is trapped from the circulation (Feld and Hirschberg, 1996; D'Ercole et al., 1984; Mathews et al., 1986). In normal rat renal tubules hardly any IGF-I mRNA is detected (Matejka et al., 1992; Matejka and Jennische, 1992), while the protein is clearly found in proximal tubules (Kobayashi et al., 1991).

Although IGF system expression in the kidney has been described in several studies (Lindenbergh-Kortleve et al., 1997; Rabkin et al., 1995; Price et al., 1995; Chin et al., 1992), descriptions of changed IGF system expression patterns in renal disease models are scarce. Another complicating factor is the fact that IGF system expression in rat differs from the pattern in mice, especially for IGFBP-1.

Ultimately, however, it is the protein that will determine the final effect on the kidney. In this regard, we would like to emphasize our finding that the similar effects of IGFBP-1 and IGF-I + IGFBP-1 on renal protein localization of the IGF system components explain the identical morphological results of both treatments, as is summarized in Figure 8.

Furthermore, we consider the effects of GH administration on kidney morphology to be comparable, but less than the effects of IGF-I + IGFBP-1 or IGFBP-1. Therefore, we examined the similarities in IGF system protein patterns between these three groups. Thus, increase in IGFBP-4 in the thick limbs of Henle's Loop, which is seen after both IGFBP-1 and IGF-I + IGFBP-1 treatment, may play an important role in the onset of fluid

accumulation and/or formation of dilatated thin limbs of Henle's Loop. The same might apply to increased IGFBP-5 in the proximal tubules and the decrease in type I IGF receptor in the distal/collecting tubules. Apparently these few changes in IGF system protein expression are not enough to result in fluid accumulation and/or dilatation of the thin limbs of Henle's Loop, within 4 weeks of GH treatment. This fluid accumulation and these dilatations are probably the result of a complex network of interacting factors (IGF system members and likely other factors), as suggested by the changed protein patterns in the kidneys of IGFBP-1 and IGF-I + IGFBP-1 treated mice.

We suggest that systemic IGFBP-1 administration mediates specific local effects in the kidney, acting directly on specific parts of the nephron (possibly via integrins). This may lead to changed fluid transport and as a consequence, fluid accumulation occurs in the thin limbs of Henle's Loop. The precise mechanism of these changes are unclear as the role of the specific changes in the expression of the IGF system components remains to be elucidated.

Summarizing, we have shown that treatment with IGFBP-1 and IGF-I + IGFBP-1 of Snell dwarf mice causes specific morphological changes in kidney. Furthermore, specific alterations on mRNA as well on protein levels of the IGF system occur. We suggest that direct, local IGFBP-1 effects on renal fluid exchange may cause dilatations of the thin limbs of Henle's Loop. Hence, our study provides a framework for a further understanding of the role of IGFBP-1 in renal physiology.

	serum IGF-I		serum IGF-I +IGFBP-I5		serum IGFBP-I5
effect on IGF system kidney mRNAs	mRNA changes	=	mRNA changes	≠	mRNA changes
effect on IGF system kidney protein	protein changes	≠	protein changes	=	protein changes
effect on kidney morphology	hyperplasia		dilatation and fluid accumulation	=	dilatation and fluid accumulation

Figure 8
Scheme to summarize the different changes in IGF system mRNA and protein after administration of IGF-I, IGF-I + IGFBP-1 or IGFBP-1. IGF-I administration results in renal hyperplasia, while administration of IGF-I + IGFBP-1 or IGFBP-1 alone result in enlarged kidneys that show dilatated thin limbs of Henle's Loop. Changes in mRNA expression are identical after administration of IGF-I and IGF-I + IGFBP-1, while identical changes in protein expression is obtained after administration of IGFBP-1 and IGF-I + IGFBP-1. Thus, changes at the protein level seem to determine the effect on renal morphology. In addition, it appears that IGFBP-1 may be responsible for the resulting fluid accumulation and dilatation.

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General Discussion

7

General discussion

Aim of this thesis

The insulin-like growth factors, their receptors, and the IGFBPs form a complex system, that is important in particular during development. The IGF system components show spatial and temporal specific expression patterns. In addition, as the IGFs and IGFBPs are secreted proteins, protein localization studies will provide a useful contribution. This thesis focuses on the role of the IGF system during growth and differentiation of the mouse, specifically by studying localization of components of the IGF system, at both the mRNA and protein levels, during mouse development. In addition, the effects of supranormal circulating levels of IGF system compounds are studied postnatally in the Snell dwarf mouse. These results will be discussed in a wider context hereafter, and also suggestions for future research will be mentioned.

Expression of the IGF system during mouse development

IGF-I and -II are both expressed at a very early stage of embryonic mouse development, at the pre-implantation phase (Heyner et al., 1989; Rappolee et al., 1990; Schultz et al., 1992). IGFBPs appear at least at 9.5 dpc and probably much earlier. Although techniques have expanded from Northern blots to *in situ* hybridization, the physiological relevance of the spatio-temporal expression of the IGF system members remains largely unknown.

The embryonic developing vertebrate limb is a useful model to study tissue outgrowth and pattern formation. In the early limb bud several important signalling centers can be distinguished that contribute to the regulation of limb bud outgrowth and patterning (Tickle, 1996). The early limb bud also requires various other signals to differentiate. The IGFs serve as ubiquitous cellular growth promoters and are modulated by the IGFBPs. Results presented in Chapter 2 show that all IGF system members, except IGFBP-1 and -6 that are below detection limits, have specific spatio-temporal mRNA expression patterns in the developing mouse limb. IGFBP-2 and -5 are found in the apical ectodermal ridge (AER), while IGF-I and IGFBP-4 are located in another signalling centre, the zone of polarizing activity (ZPA). These findings suggest a function for the IGF system in limb bud differentiation. Furthermore, localization of IGF-II and the type I IGF receptor in pre-cartilage supports the hypothesis that the IGF system is involved in chondrogenesis. A more general function for the IGFBPs as auto- or paracrine regulators of IGF action during mouse development is described in Chapter 4. Whether IGF-independent actions of the IGFBPs, as suggested for the adult mouse (Chapter 6), are also operative in the mouse embryo, is not clear.

IGF system expression and apoptosis during mouse development

The expression of IGFBP-2, -4 and -5 in the interdigital zone of the developing limb colocalizes IGF system expression with apoptosis (van Kleffens et al., 1998; Chapter 2). Especially IGFBP-2 is expressed in the same area where apoptotic cells are found. IGFBP-3, -4 and -5 are also expressed in areas with apoptosis, in the phalangeal joint areas, at an early stage of joint formation.

Analysis of regions with apoptosis in the whole mouse embryo at different time points, as described in Chapter 3, provides further evidence that expression of the IGF system is correlated with regions with apoptosis. For example, after decrease of IGF-I expression in

the developing whisker, apoptosis in the same areas occurs. This may indicate confirmation of the proposed protective function of IGF-I in apoptosis.

Despite the likely correlation of IGF-I with apoptosis, the general conclusion of these descriptive studies is that it is impossible to predict which subset of IGF system components will be active in a given area with apoptotic cell death. The complexity of this system suggests that this is a temporally and spatially regulated interaction, not only between the IGF system members, but between the IGF system and other (growth) factors as well (see below).

Correlation of mRNA expression of the IGF system with protein localization

As is shown in Chapters 3, 4 and 6, mRNA and protein localization of the IGFs and their binding proteins are not completely overlapping. Differences were specifically found for IGFBP-3, 5, -6 (Chapter 3). In general, this can be explained by the fact that the IGF system components, except the IGF receptors, are secreted proteins. Therefore, existing data on mRNA location of the IGF system components do not necessarily completely overlap protein localization. With the IGFBP-antibodies that now have become available (Chapter 3), localization the IGF system can be completed at the protein level. In addition, these antibodies can be applied for example in studies with transgenic mice, in pharmacological studies, in organ culture experiments, and to a limited extent also in experiments that involve Westernblotting. Thus, a framework is available for further characterization of IGF system regulation.

Effects of administration of IGF-I, IGFBP-1 or IGF-I + IGFBP-1

After determination of mRNA and protein expression patterns of the IGF system in the normal situation, we choose to study the effects of manipulation of serum levels of the IGF system components in the Snell dwarf mouse, postnatally (Chapters 5 and 6). This mouse is GH deficient, and thus lacks any feedback between GH and IGF system members. IGF-I production in liver is lacking, and serum IGF-I levels are very low. This mouse is a good model to study the effects of administration of IGF system compounds, without the complex interference of feedback mechanisms. These studies clearly demonstrate the dual effect of IGFBP-1: inhibiting IGF-I-stimulated body growth, while stimulating kidney growth (Chapter 5). Characterization of the morphological renal effects, and of the effects on mRNA and protein expression and localization, showed a local function for IGFBP-1 in the kidney. This is a good example of the advantage of the application of IGFBP-antibodies. The changes in mRNA expression did not give any clue in understanding the morphological changes seen upon treatment with IGFBP-1 and IGF-I + IGFBP-1. Surprisingly, changes in protein localization after these treatments were exactly similar. The exact mechanism of action, however, awaits to be elucidated.

Future directions

Interaction with other (growth) factors

From all studies focused on elucidating the role of the IGF system during development, some conclusions can be drawn. Tissue outgrowth and pattern formation during development is the result of a complex network of interactions (Tickle, 1996). Thus, it is relevant to correlate the IGF system expression patterns to those of other genes involved in development, to get a better insight into the possible interactions between various gene products. For example, interactions between the epidermal growth factor (EGF) and IGF system have been suggested (Hana and Murphy, 1994). In addition, IGFBP-3 has been shown to mediate transforming growth factor-β (TGF-β), retinoic acid (RA)-, and antiestrogen-induced growth inhibition in breast cancer cells (Oh et al., 1995; Huynh et al., 1996; Gucev et al., 1996).

In addition, the human IGFBP genes are in close proximity to the homeobox gene clusters (Hox-A through Hox-D) and seem to have evolved together. It has been suggested that these genes have originated from single genes, which underwent several coordinate duplications and translocations (Collet-Solberg and Cohen, 1996). Hox genes encode DNA binding proteins, and can be transcriptionally regulated by retinoic acid, like some of the IGFBP genes. In our study, expression patterns of some of the IGFBP mRNAs in the developing mouse limb appeared to be partly similar to the reported localization of specific Hox mRNAs. This suggests a possible link between these gene families. Several Hox deficient mice have been reported, which show varying developmental disorders (Muragaki et al., 1996; Zakany and Duboule, 1996). It would be of interest to analyse whether the expression patterns of the IGF system in specific regions of these knockout mice is changed and whether this is correlated with changes in expression patterns of other genes.

Epithelio-mesenchymal interactions

Epithelial-mesenchymal interactions and the possible involvement of the IGF system in combination with other factors deserves attention. It is obvious that a crosstalk exists between these cell layers in many organs. In some tissues, such as limb (Chapter 2), but also in lung and kidney (Chapter 3), the IGFs and their binding proteins are found located in neighbouring cell layers, often including the epithelium. The distinct spatial expression of the IGF system suggests an effect of this system on facilitation of epithelio-mesenchymal cell interaction. The prevailing idea is that the endocrine actions of the IGF system, acting through the circulation, are fine-tuned by locally produced members of the IGF system. An isolated system of tissues or organs in culture could give more insight in this phenomenon (see also below).

IGF independent actions of the IGFBPs

Not all IGFBP actions are necessarily through IGF-receptor interaction. It has already been shown that IGFBP-1 and -3 have direct effects on cell growth, and such actions are also postulated for IGFBP-2, -4 and -5 (Cohen et al., 1993; Oh et al., 1993a/b). The IGF independent actions relate to the cell surface binding capacity of the IGFBPs. IGFBP-1 has a RGD sequence, known to be recognized by the integrin receptors on the cell membrane (Ruoshlati and Pierschlaber, 1987). For IGFBP-2, which also has an Arg-Gly-Asp (RGD) sequence, such integrin-type receptor binding has not been demonstrated. However,

IGFBP-2 is cell surface-associated via other unknown mechanisms (Cohen et al., 1995). IGFBP-3 and -5 bind to endothelial cell monolayers and are found in large concentrations in the extracellular matrix. Binding of IGFBP-4 to cell membranes has been demonstrated, the function of which is unknown (Hasegawa et al., 1995). Until now, studies on IGFBP-1, -3 and -5 action suggest possible non-IGF-mediated actions of these binding proteins (Jones et al., 1993; Jones and Clemmons, 1995; Andress, 1995). IGFBP-3 IGF-independent actions have been characterized most extensively, and have been shown to be anti-proliferative (Oh et al., 1993b). However, IGFBP-receptors and molecular mechanisms need to be elucidated. It may be useful to try to characterize these receptors and their signalling using two-hybrid systems (yeast-based genetic assays for detection of protein-protein interactions) (Gyuris et al., 1987; Fields, 1989). In this *in vivo* system, weak and transient interactions can be detected. A yeast two-hybrid system where the IGFBPs are used as a 'bait' could possibly result in characterisation of the IGFBP receptors. The same system could then elucidate the mechanism of IGFBP signalling via their own receptors.

Gene manipulation studies

To understand the IGF-independent actions of the IGFBPs and other functions of the IGF system, gene manipulation studies are highly relevant. The knockout mice carrying null mutations of the IGFBPs do not show abnormal phenotypes (see general introduction). Therefore, tissue-specific gene mutation (conditioned knockout) or overexpression may give further clues. In addition, regulation by other genes or gene-regulatory elements can be studied, by integrating the sequences of interest in artificial chromosomes. The ability of integrating large DNA sequences in these artificial chromosomes is of great importance in the construction of transgenic animals.

In vitro manipulation studies (organ cultures)

Finally, organ or tissue culture systems, in which the expression of genes is manipulated by making use of anti-sense oligonucleotides, or various proteins, may play a role in solving questions regarding tissue-specific actions of the IGF system during development. However, until now the antisense technology still has to deal with many pitfalls. One of the most important aspects is to prove complete penetration of the oligonucleotide and to monitor its activity in the organ or tissue of interest.

An alternative, the use of specific antibodies to block protein action, is now becoming a reasonable possibility, since specific IGFBP antibodies were developed, as described in Chapter 3.

Together, these studies will lead to a better understanding of the roles of IGF system components. The complexity of the IGF system makes such research difficult and laborious, but the function of the IGF system in pathogenesis of various diseases is of sufficient interest to warrant such an effort.

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Summary

8

Summary

Summary

The insulin-like growth factors (IGF)-I and -II are low molecular weight single chain polypeptides. The IGFs promote mitosis and differentiation of a variety of cell types, and have been particularly implicated in fetal and placental growth. The IGFs mediate their effects by binding to two specific IGF receptors on target cell surfaces. The IGFs circulate bound to a family of binding proteins. At least six IGFBPs have been characterized, designated IGFBP-1 through -6. The IGFBPs are structurally related and modulate IGF action in a spatial and temporal specific manner.

In this thesis, localization studies and *in vivo* administration have been performed to get more information on the time and tissue dependent action of the IGF system components.

Chapter 2 describes the mRNA expression patterns of IGF system components in the developing mouse limb. Using whole mount *in situ* hybridization of mouse limbs at 9.5, 11.5 and 13.5 days post coitum (dpc), it was shown that the IGF system components, except IGFBP-1 and -6 (which, if expressed, are below detection limits), show spatial and temporal specific expression patterns. The 9.5 dpc limb bud contains several important signalling centres that contribute to the regulation of limb bud outgrowth and patterning. IGFBP-2 and -5 were localized in the apical ectodermal ridge (AER), and IGF-I and IGFBP-4 in the zone of polarizing activity (ZPA). In the 13.5 dpc limb bud strong tissue remodelling takes place. The interdigital tissue disappears through apoptosis and pre-cartilage of the future digits is formed. IGFBP-2, -4 and -5 mRNAs were found in the interdigital zone, while IGFBP-3 and IGF-I bordered this region. IGF-II and the type I IGF receptor genes were expressed in the pre-cartilage, while IGFBP-3, -4 and -5 mRNAs were found in the phalangeal joint areas, at an early stage of joint formation. These results point to a possible involvement of the IGF system in chondrogenic differentiation of mesenchyme and the regulation of apoptosis in the developing limb.

As the IGFBPs are secreted proteins, it is interesting to know the localization of both mRNA and the resulting protein. In Chapter 3 the production and characterisation of six different polyclonal antibodies directed against the six IGFBPs is described. The specificity of these antibodies was shown with ELISA, Westernblotting and immunohistochemistry. These antibodies were employed to localize IGFBP proteins in the mouse embryo. Next, protein patterns were correlated with mRNA expression patterns in the mouse embryo, as obtained with in situ mRNA hybridization. Protein and mRNA expression patterns of the IGFBPs in the 13.5 dpc mouse embryo were tissue-specific. Like IGFBP-1 mRNA, IGFBP-1 protein was only found in liver. The distribution of IGFBP-2, -4 and -5 protein and mRNA was more widespread, and these were found in various organs and tissues. IGFBP-3 and -6 protein and mRNA levels were low in the 13.5 dpc mouse embryo. Several organs and tissues contained more than one IGFBP. These IGFBPs may each have a different, specific function. On the other hand, the IGFBPs may show some functional redundancy. Differences between mRNA and protein expression were mainly found for IGFBP-3, -5 and -6. This may be explained by the secretion and transport of these IGFBPs. On the other hand, the general overlapping cellular mRNA and protein localization of the IGFBPs indicate that they may act as auto- or paracrine regulators of IGF action during mouse development.

Analysis of the correlation of mRNA and protein patterns of the IGF system components, together with cell death patterns in the mouse embryo is described in Chapter 4. *In situ* hybridization (mRNA), immunohistochemistry (protein) and Tunel (cell death) techniques were used on sections of 9.5, 11.5 and 13.5 dpc mouse embryos. The areas analyzed are characterized by the occurrence of apoptotic cell death. Apoptotic cell death in the developing embryo contributes to tissue remodelling. This study provided further confirmation of the involvement of the IGF system in apoptosis. It was shown that in all areas with apoptosis studied, the IGF system was expressed spatially and temporally specific. In the eye muscle, somites, and whiskers, decrease of IGF system expression was followed by the occurrence of apoptic cells. Therefore, these results confirm the protective function of the IGF system against apoptosis. Time and site dependent IGFBP expression may function as specific modulation of inhibiting actions of IGF on apoptosis. Thus, the IGFBPs may contribute to specific tissue modelling during mouse development.

In the next chapters emphasis was put on the effects of additional circulatory IGFBP-1 on IGF-I induced somatic and organ growth of the Snell dwarf mouse. This mouse is Pit-1 deficient and lacks endogenous GH and IGF-I. In the first part (Chapter 5) the effects of administration on body and organ growth is described. Human growth hormone, IGF-I and IGFBP-1, and a pre-equilibrated combination of equimolar amounts of IGF-I and IGFBP-1 were administered subcutaneously during 4 weeks. Treatment with IGF-I increased body length and weight, as well as the weight of several organs. IGFBP-1 administration increased kidney weight, but inhibited thymus growth. Combination of IGF-I with IGFBP-1 neutralized most of the IGF-I stimulating effects, except for growth of kidney, submandibular glands and spleen. Analysis of the sera showed slower clearence of IGFBP-1 from the circulation in absence of IGF-I. These results are very interesting and warrant further investigation, since elevated IGFBP-1 levels occur in several disease states.

The kidney specific effects of IGF-I + IGFBP-1 and IGFBP-1 administration were further investigated as described in Chapter 6. Morphological analysis of kidneys of treated Snell dwarf mice showed that the stimulating effect of IGF-I treatment on kidney weight was caused by cellular proliferation. However, the enlarged kidneys after IGF-I + IGFBP-1 and IGFBP-1 administration were the result of dilatated thin limbs of Henle's Loop and fluid accumulation. Further characterization of these kidneys was done by Northernblotting, *in situ* hybridization, and immunohistochemistry. The studied mRNA and protein expression patterns were changed by all treatment regimens. IGF-I and IGF-I + IGFBP-1 treatments gave the same mRNA changes. Protein patterns, however, were influenced similarly by administration of either IGFBP-1 or IGF-I + IGFBP-1. Although the exact mechanism remains to be elucidated, it may be concluded that IGFBP-1 inhibits IGF-I effects on mRNA and protein expression. Furthermore, IGFBP-1 may function in an IGF-independent manner in the kidney, causing local changes in tissue morphology and cell proliferation, probably via changes in mRNA and protein expression of the IGF system.

In the general discussion (Chapter 7), findings of these studies are discussed in a wider context. In addition, examples for further research on these and related topics are given. Summarized, the mRNA localization studies, combined with studies on protein localization with the newly developed IGFBP-antibodies, have enabled a framework for further research on functioning of the IGF system during growth and differentiation of the mouse. This work is relevant for our understanding of (dys)regulation of growth and development, also in other mammalian species including the human.

8

Summary

Chapter 9

Populair wetenschappelijke samenvatting

9

Populair wetenschappelijke samenvating

Populair wetenschappelijke samenvatting

Dit proefschrift beschrijft onderzoek aan insuline-achtige groeifactoren (IGF-I en -II) en daaraan gerelateerde eiwitten. IGFs zijn kleine eiwitten, die een belangrijke rol spelen bij regulatie van proliferatie, differentiatie en afsterven van cellen. Het merendeel van de IGFs wordt aangemaakt in de lever, door middel van een signaal dat via het groeihormoon (GH) vanuit de hypofyse komt. Vervolgens komen deze groeifactoren in het bloed terecht, waar zij binden aan IGF-bindende eiwitten (IGFBPs). Van deze IGFBPs bestaan tenminste zes verschillende soorten, die erg op elkaar lijken qua structuur (IGFBP-1 t/m -6). De IGFBPs worden door allerlei verschillende organen en celtypen geproduceerd. Door aan IGF te binden zijn ze in staat de werking van IGF te beïnvloeden. Enerzijds fungeren de IGFBPs als transporteurs van IGF naar het orgaan of celtype van bestemming. Hier kan IGF vervolgens binden aan zogeheten IGF receptoren die in de buitenmembraan van de cel verankerd zijn. Deze receptoren geven vervolgens een signaal door naar de kern van de cel. Anderzijds kunnen de IGFBPs de werking van IGF belemmeren, doordat IGF dat gebonden is aan de IGFBPs niet bij de receptor komt. Recent onderzoek heeft aangetoond dat IGFBPs ook kunnen functioneren zonder aan IGF te binden, met een eigen receptor.

Eén van de belangrijkste functies van dit complexe systeem van IGFs en IGF-gerelateerde eiwitten is het reguleren van groei tijdens de embryonale ontwikkeling. De experimenten die in dit proefschrift zijn beschreven zijn gericht op de lokalisatie van het IGF systeem tijdens de ontwikkeling van het muizenembryo. Dit kan aanwijzingen geven over de mogelijke functie(s) van het IGF systeem. Gekeken is naar zowel het voorkomen van eiwit, als van het boodschapper RNA (messenger ribo-nucleic acid, mRNA). mRNA is het tussenprodukt dat ontstaat als een gen actief wordt (tot expressie komt) en dat vertaald wordt naar eiwit. In tegenstelling tot het mRNA, blijven de IGFs en IGFBP eiwitten niet in de cel, maar worden uitgescheiden en kunnen dus vrij circuleren en in het weefsel andere cellen bereiken of via het bloed in allerlei organen komen.

Na het inleidend hoofdstuk, waarin achtergrondinformatie over het IGF systeem wordt gegeven en het doel van dit proefschrift wordt uiteengezet, volgt Hoofdstuk 2. Hierin wordt beschreven in welke delen van een muizenpootje (hand en voet) tijdens de ontwikkeling het mRNA van de diverse componenten van het IGF systeem voorkomt. Een muizenpootje tijdens de ontwikkeling is een goed modelsysteem voor het bestuderen van celproliferatie en differentiatie, omdat het in korte tijd grote veranderingen ondergaat. Het begint als knopje op 9.5 dag na de coïtus (dpc) en op 11.5 dpc is er een hand/voetplaat zichtbaar (zie Hoofdstuk 2, Figuur 1). Op 13.5 dpc is het vervolgens uitgegroeid tot een voetje, en afzonderlijke tenen. Voor het vormen van de tenen, waarbij tussenliggend weefsel verdwijnt, treedt een proces op dat geprogrammeerde celdood (apoptose) wordt genoemd.

Uit de resultaten blijkt dat de verschillende genen van het IGF systeem op een specifieke manier, qua tijd en plaats, tot expressie komen. In het vroege ontwikkelingsstadium van het pootje valt de localisatie van IGF-I en IGFBP-4 samen met een belangrijk signaalgebied in het pootje (zone met polariserende activiteit, ZPA). Een ander signaalgebied is de buitenste rand van het pootje, de apicale ectodermale richel (AER), waarin IGFBP-2 en -5 worden aangetoond. In een later stadium blijken de genen voor IGF-II en de IGF receptor tot expressie te komen in het zich vormende kraakbeen.

Tenslotte wordt IGFBP-2, -4 en -5 mRNA aangetoond in het gebied tussen de tenen.
Deze karakteristieke mRNA patronen leidden tot de hypotheses dat het IGF systeem betrokken is bij de differentiatie van mesenchym cellen naar botvormende cellen en bij de regulatie van apoptose in het ontwikkelende pootje.

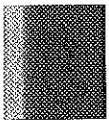
In het volgende Hoofdstuk (Hoofdstuk 3) ligt de nadruk op het aantonen van eiwit van de IGFBPs. Om dit te kunnen werden speciale antilichamen ontwikkeld. Deze antilichamen herkennen het specifieke IGFBP-eiwit, binden eraan en vervolgens kan met een speciale techniek deze binding zichtbaar worden gemaakt. Het ontwikkelen en testen van deze antilichamen wordt beschreven, plus de toepassing in het muizenembryo. Hiervoor werd een embryo gefixeerd en ingebed in paraffine. Hiervan werden plakjes (coupes) gesneden van 5µm, die vervolgens op glaasjes werden geplakt. Het aantonen van eiwitten met behulp van antilichamen op coupes wordt immunohistochemie genoemd. Er werd aangetoond dat er grote overeenkomsten tussen de lokalisatie van mRNA en eiwit van de afzonderlijke IGFBPs bestaan. Verder werd bevestigd dat de IGFBPs allemaal in specifieke organen en weefsels voorkomen, elk met een karakteristiek patroon. Deze resultaten geven aan dat de IGFBPs waarschijnlijk de werking van de groeifactoren ter plekke (lokaal) reguleren.
Hoofdstuk 4 gaat nog iets verder in op het vergelijken van lokalisatie van eiwit en mRNA. Er werd gekeken naar gebieden in muizenembryo's met veel apoptose en er werd geprobeerd een correlatie te vinden tussen mRNA en eiwitpatronen van het IGF-systeem en de plaats van apoptotische cellen. Uit de resultaten werd afgeleid dat er inderdaad overeenkomsten bestaan tussen het voorkomen van het IGF-systeem en apoptose. Alleen bleek dit voor elk bestudeerd gebied een andere combinatie van eiwitten te zijn. In het algemeen geldt dat IGF-I en de IGF-I receptor remmend lijken te werken op het optreden van apoptose. Het zou zo kunnen zijn dat specifieke regulatie van deze functies van IGF en de IGF-I receptor in elk weefsel door een andere (set van) IGFBP plaats vindt. Hierdoor zouden de IGFBPs bij kunnen dragen aan de specifieke verandering van weefsels tijdens de ontwikkeling van de muis.

In Hoofdstukken 5 en 6 worden de effecten van extra IGFBP-1 in bloed bekeken, bij muizen die door IGF-I behandeling grotere afmetingen en organen hebben gekregen. GH dat normaal gesproken een wisselwerking met IGF-I heeft, is in deze speciale (dwerg) muis niet aanwezig. Eerst worden in Hoofdstuk 5 de effecten op de groei van het lichaam en de organen beschreven. Na een behandeling van 4 weken met IGF-I waren lengte en gewicht toegenomen, net als het gewicht van verschillende organen. Behandeling met IGFBP-1 zorgde voor toename van het niergewicht, maar remde de groei van de thymus. Behandeling met een combinatie van IGF-I en IGFBP-1 liet behalve groei van nier, speekselklieren en milt, geen van de effecten zien die door behandeling met IGF-I alleen wel werden veroorzaakt. Deze specifieke IGFBP-1 effecten zijn interessant voor verdere studies, aangezien een verhoogde hoeveelheid IGFBP-1 in bloed bij sommige ziekten voorkomt.

Vervolgens werd het effect van IGFBP-1 op niergroei nader bestudeerd in Hoofdstuk 6. Eerst werd gekeken naar de cellulaire structuur (morfologie) van de behandelde nieren. De nieren bleken bij behandeling met IGF-I vergroot te zijn doordat er cellen bijgekomen waren (proliferatie). Bij de behandeling met IGFBP-1 en IGF-I + IGFBP-1 werd iets heel

anders waargenomen: hier waren bepaalde nierbuisjes (de dunne 'loop van Henle') opgezet. Vervolgens werd de invloed van behandeling op genactiviteit bekeken, zowel wat betreft mRNA als eiwit. De hoeveelheid en plaats van mRNA en eiwit bleken inderdaad te veranderen door de verschillende behandelingen. De behandelingen met IGF-I en IGF-I + IGFBP-1 resulteerden in dezelfde mRNA veranderingen. Voor eiwit was dit anders, want in dit geval waren de veranderingen gelijk bij de behandelingen met IGFBP-1 en IGF-I + IGFBP-1. Hoewel hiermee het exacte mechanisme nog niet is opgehelderd, kan toch vastgesteld worden dat IGFBP-1 remmend werkt op IGF-I effecten. Verder zou IGFBP-1 onafhankelijk van IGF-I in de nier werkzaam kunnen zijn. Hiermee zouden de veranderingen in de nier, qua structuur en qua genexpressie verklaard kunnen worden.

In de algemene discussie (Hoofdstuk 7) worden al deze resultaten en conclusies in een bredere context besproken. Ook worden er aanbevelingen gedaan voor toekomstig onderzoek. Samengevat vormen de mRNA studies, gecombineerd met de detectie van eiwit met de ontwikkelde antilichamen een goede basis voor verder onderzoek naar de functies van het IGF systeem tijdens groei en differentiatie van de muis. Dit werk is van belang voor het begrip van (dys)regulatie van groei en ontwikkeling, ook in andere zoogdieren, inclusief de mens.



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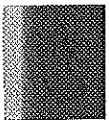

List of publications

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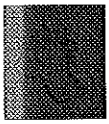

Dankwoord

Poeh poeh, hè hè, nou nou, het boekje is af! De laatste loodjes wogen wel wat kilo's, maar gezien de verhuizing vlak erna was dit misschien wel een hele goede training. Cliché, cliché, maar ook dit 'boekje' was er niet geweest zonder alle hulp van heel veel mensen, gedurende de afgelopen jaren. Met het risico dat ik sommige mensen vergeet, ga ik toch wat namen noemen.

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Haje,

Marjolein