MODULATION OF VITAMIN D ACTION BY LOCAL FACTORS IN BONE

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MODULATIE VAN DE WERKING VAN VITAMINE D DOOR LOKALE FACTOREN IN BOT

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam op gezag van de
rector magnificus Prof. Dr. P.W.C. Akkermans M.A.
en volgens besluit van het College van Promoties.
De openbare verdediging zal plaatsvinden op
woensdag 26 juni 1996 om 11.45 uur.

door

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geboren te Vlaardingen

Promotiecommissie

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The investigations described in this thesis were performed at the Department of Internal Medicine III of the Erasmus University Medical School, Rotterdam, The Netherlands and in part at the Department of Cell Biology of the University of Massachusetts Medical Center, Worcester, Massachusetts, USA. The studies were supported by a grant of the Netherlands Organization for Scientific Research (NWO Project no. 900-541-131).

Cover picture:
Microscopic magnification of cortical bone.
Inset: Microscopic magnification of trabecular bone.

In memory of my father

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CHAPTER 1

INTRODUCTION

1.1 BONE

1.1.1 A dynamic tissue

Bone provides rigid mechanical support, protects vital organs and serves as a reservoir of ions, such as calcium and phosphate, that can be mobilized from bone. There are two types of bone: cortical bone, which is composed of densely packed, mineralized collagen laid down in layers and provides rigidity, and trabecular bone which is spongy in appearance, and also consists of mineralized collagen and provides strength and elasticity. There are also two types of bone formation: intramembranous ossification, which primarily takes place in the calvarium and starts within the connective tissue, and endochondral ossification, which takes place in long bones and starts with the formation of cartilage followed by bone formation and mineralization. The mineralized matrix is composed of collagenous and noncollagenous proteins and contains a variety of locally produced growth factors.

The integrity of the skeleton is maintained by continuous remodeling of bone that occurs throughout life. The three major types of bone cells are the osteoblasts, osteocytes and osteoclasts. Osteoblasts are bone forming cells and lie together with the lining cells, which are inactive osteoblasts, on the endosteal surfaces of bone. The osteoblasts form new bone on previously resorbed bone, by synthesizing bone matrix (osteoid), which subsequently mineralizes. Osteoblasts are derived from local mesenchymal tissue. Osteocytes are osteoblasts, that have been trapped in the mineralized matrix and have changed their functional and morphologic characteristics. They have multiple processes that reach out through lacunae in bone tissue, and may have a mechanosensor function in bone. Osteoclasts, bone resorbing cells, are multinucleated cells which are responsible for bone resorption. Osteoclasts are derived from circulating mononucleated hematopoietic precursors, although their precise origin is still unknown. Proliferation and differentiation of skeletal cells are influenced by many factors, including systemic hormones, like 1,25-dihydroxyvitamin D_3 and parathyroid hormone (PTH), as well as locally produced factors. Linkage of bone formation to bone resorption is called "coupling". Under normal physiological conditions, in the remodeling process bone formation and resorption are strictly coupled. In pathophysiological situations there is a disbalance, e.g. in case of osteoporosis there is more resorption in relation to formation, and it is conceivable that locally produced growth factors play an important role as mediators in the coupling process (Figure 1).

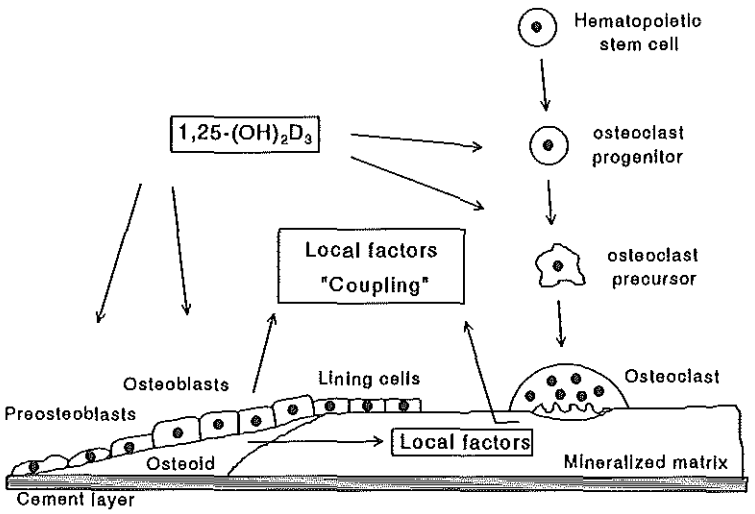

Figure 1

Schematic representation of the proposed role of local factors in coupling bone formation to bone resorption. Development of osteoblasts and osteoclasts from their precursors is affected by systemic hormones, like 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$). Upon activation by 1,25-$(OH)_2D_3$, osteoblasts synthesize factors, which may either directly affect osteoblast and/or osteoclast function or may be stored in the bone matrix. Local factors stored in the bone matrix may be released and activated during bone resorption by osteoclasts and subsequently affect osteoblast and/or osteoclast function. Lining cells are inactive osteoblasts.

1.1.2 Bone matrix proteins

The primary component of the organic bone matrix is collagen, which is essentially collagen type I. Furthermore, several non-collagenous bone matrix proteins have been identified. The most important non-collagenous bone matrix proteins are summarized in Table 1. In view of the present thesis two of these proteins, osteocalcin and osteopontin will be discussed more extensively.

1.1.3 Osteocalcin

Osteocalcin represents 15-20% of the non-collagenous proteins in the bone matrix. The osteocalcin gene consists of four exons and three introns from which a low molecular weight protein of 5.8 kD is synthesized which consists of 49 amino acid residues after cleavage of a prepropeptide of 10 kD. Vitamin K is required for the

TABLE 1

Most important non-collagenous bone matrix proteins, subdivided according to structure.

Noncollagenous bone	matrix proteins
Poteoglycans and hyaluronan	Chondroitin sulfate proteoglcan
	Biglycan
	Decorin
Glycoproteins	Alkaline phosphatase
	Osteonectin
	Bone acidic glycoprotein-75
RGD containing proteins	Fibronectin
	Vitronectin
	Osteopontin
	Bone sialoprotein
	Thrombospondin
Gla containing proteins	Osteocalcin
	Matrix Gla protein
	Protein S
	Growth factors Proteolipids Serum proteins

carboxylation of osteocalcin, which is catalyzed by the vitamin K-dependent carboxylase, the enzyme responsible for the posttranslational conversion of three glutamyl (Glu) to gamma-carboxyglutamyl (Gla) residues (1,2). Osteocalcin has a high affinity for Ca^{2+}, due to the carboxylated residues.

Osteocalcin is expressed only postproliferatively in the mature osteoblast at the onset of extracellular matrix mineralization (3) (Figure 2). Furthermore, odontoblasts have been shown to produce osteocalcin (4). Recently, osteocalcin production has been shown in cells isolated from bovine aortic media, which show osteoblastic potential (5), and in bone marrow megakaryocytes and peripheral blood platelets (6).

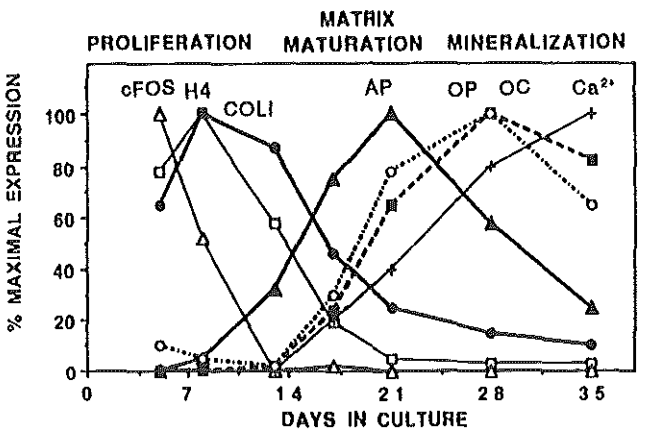

Figure 2

Temporal expression of cell growth and osteoblast phenotype related genes during the development of *in vitro* formed bone-like tissue by normal diploid rat osteoblasts as reported by Stein and Lian (3). The proliferation period is characterized by the expression of c-fos, histone H4 and collagen type I (Coll). Alkaline phosphatase (AP) is expressed postproliferatively and associated with extracellular matrix maturation. With the onset of extracellular matrix mineralization, osteopontin (OP) and osteocalcin (OC) are expressed, and calcium (Ca^{2+}) accumulates in the extracellular matrix.

The exact function of osteocalcin in bone is unknown, but a role of osteocalcin in bone resorption has been suggested. Warfarin, which inhibits the carboxylation of osteocalcin and reduces the amount of osteocalcin in bone, probably as a result of the lack of calcium binding Gla residues, has been used in several studies in which the role of osteocalcin in bone was investigated. In warfarin-treated lambs, a reduction in bone turnover is observed (7) and from results obtained by using warfarin-treated bone particles, a role for osteocalcin has been suggested in the recruitment and/or differentiation of bone resorbing cells (8-10). Furthermore, osteocalcin is a chemo-attractant of human peripheral blood monocytes and macrophages (11-13). The mineral binding properties of osteocalcin as well as its pattern of expression suggest a role for osteocalcin in bone mineralization. Recently, the absence of osteocalcin has been shown to increase bone mass in osteocalcin deficient mice (14). A small fraction of osteocalcin is released into the circulation and is used as a marker of bone turnover *in vivo*.

The production of osteocalcin is regulated by steroid hormones and local factors in bone, including 1,25-$(OH)_2D_3$ (15-17), glucocorticoids (17-19), estrogen (20,21),

thyroid hormone (22,23), PTH (17,24,25), TGFß (26), basic and acidic FGF (27) and IGF-I (28,29). In the promoter region of the rat osteocalcin gene several regulatory elements, involved in basal and modulated osteocalcin gene transcription, have been characterized. These include a vitamin D response element (VDRE) (30-32), AP-1 binding sites, one overlapping the VDRE, and another one overlapping the so-called osteocalcin box, which contains CCAAT as the central motif (33,34). Furthermore, a TGFß responsive element (35), a binding site for Msx homeodomain proteins (36,37), glucocorticoid response elements (GREs) (38,39), and NMP-2 binding sites (40,41) have been identified. Stein and Lian (42) proposed a three dimensional model for the rat osteocalcin promoter in which chromatin structure and nucleosome organization play an important role in supporting crosstalk between promoter elements (Figure 3).

The human osteocalcin promoter, which has 70% sequence homology with the rat osteocalcin promoter, also contains a VDRE (43,44) with an overlapping AP-1 binding site (45,46). Furthermore, a GRE overlapping the TATA box (47,48) and a binding site for NF kappa B have been identified (49).

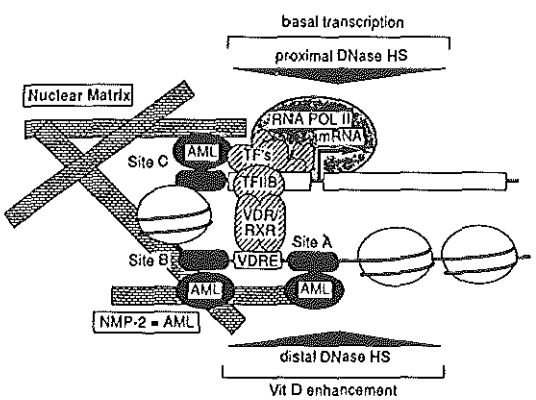

Figure 3

Model for the three dimensional organization of the osteocalcin gene promoter as proposed by Stein and Lian (42). This model shows the spatial organization of the osteocalcin promoter based on nucleosome localization and the interaction of regulatory sites with the nuclear matrix; sites A, B and C with a complex, previously designated nuclear matrix protein-2 (NMP-2), containing an acute myelogenous leukemia (AML)-related transactivation protein. Chromatin structure and nuclear architecture restrict mobility of the promoter and may reduce distances between distal and proximal promoter elements; e.g. VDR/RXR complexes binding the vitamin D response element (VDRE) and the core transcriptional machinery (transcription factor IIB = TFIIB). HS = Hypersensitive site, TFs = Transcription factors.

1.1.4 Osteopontin

Osteopontin is a phosphorylated glycoprotein that is rich in sialic acid. The osteopontin gene consists of seven exons and six introns from which a 264-301 amino acids containing protein is synthesized. The exact length depends on species. Among species, a high level of conservation of amino acid residues consists in three regions. A highly conserved region in the NH_2-terminus is thought to be a substrate for intracellular kinases responsible for catalyzing the phosphorylation of osteopontin. Near the center of the molecule is the highly conserved GRGDS sequence, which has binding properties for the cell surface $\alpha_v\beta_3$ integrins on several cell types (50). The COOH-terminal portion of osteopontin contains a non-RGD cell attachment component (51). Posttranslational modifications, including phosphorylation, glycosylation and sulfation may allow the formation of distinct forms of osteopontin (52-54).

Osteopontin is not only expressed in bone tissue, but also in several other tissues, including kidney, ovary, uterus, mammary gland, gastrointestinal tract, and dental tissue (55-58). In bone, osteopontin is synthesized and secreted by osteoblasts, osteocytes and osteoclasts. Preosteoblasts primarily synthesize the 55 kD form of osteopontin, which is a low-phosphorylated and -sulfated form, whereas the more mature osteoblasts secrete the 44 kD form of osteopontin, which is highly phosphorylated and sulfated (59). The synthesis of the 55 kD form correlates with the formation of the 'cement' layer, prior to bone deposition, whereas the 44 kD form associates with hydroxyapatite, and may be involved in regulation of crystal growth (59). In primary rat osteoblasts, osteopontin is marginally expressed during the period of active proliferation, decreases postproliferatively and is increased at the onset of extracellular matrix mineralization (3) (Figure 2).

The 44 kD osteopontin has been postulated to be involved in the osteoclast binding to the mineral surface mediated by the $\alpha_v\beta_3$ integrin and the RGD sequence in the osteopontin protein (60-62). Osteopontin is present at the bone surface facing the clear zone of osteoclasts, which is the site where the osteoclast attaches to bone. Lower amounts of osteopontin are present at the bone surface facing the ruffled border of the osteoclast, where resorption takes place. Likewise, the integrin is enriched in the clear zone of the osteoclast, but not in the ruffled border area (63).

Osteopontin synthesis by osteoblasts is regulated by several systemic and local factors, including 1,25-$(OH)_2D_3$ (64), PTH (65), estrogen (66), TGFß (67), leukemia inhibitory factor (68), platelet-derived growth factor (69) and IGF-I (70).

The osteopontin genes of several species have been cloned and putative CAAT

and TATA sequences have been found in the promoter regions (66,71,72). In the mouse osteopontin promoter, a VDRE has been identified (73), whereas, recently, in the rat osteopontin promoter a proximal and a distal VDRE have been identified and characterized (72).

1.2 VITAMIN D

1.2.1 Vitamin D metabolism

One of the major calciotrophic and bone regulatory hormones is vitamin D. The seco-steroid vitamin D_3 is either obtained from the diet or is synthesized in the skin from biologically inert 7-dehydrocholesterol under the influence of ultraviolet light. Next, vitamin D_3 is converted to the inactive hormonal precursor 25-(OH)D_3 by the enzyme 25-hydroxylase in the liver. Subsequently, the enzyme 1α-hydroxylase converts 25-(OH)D_3 to the biologically active form 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$) in the kidney (Figures 4 and 5).

Formation of the biologically active form of vitamin D_3 is tightly regulated through modulation of 1α-hydroxylase activity. PTH and reduced levels of calcium and phosphate are the most important stimulators of 1α-hydroxylase activity. Alternatively, 25-(OH)D_3 is converted to 24,25-dihydroxyvitamin D by the renal enzyme 24-hydroxylase under stimulation of 1,25-$(OH)_2D_3$. In addition, 1α-hydroxylase activity is inhibited by

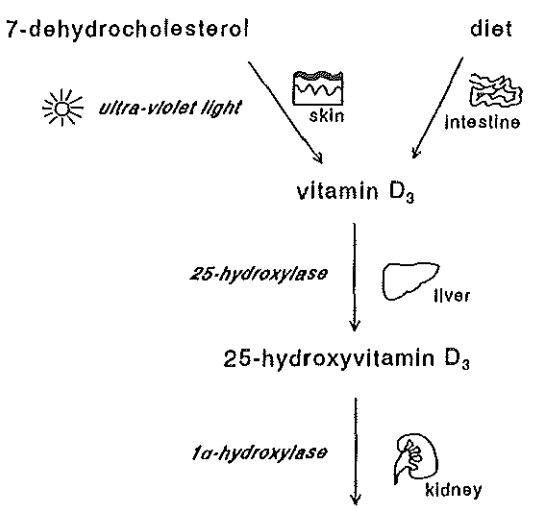

Figure 4

Formation of the biologically active form of vitamin D_3, 1,25-dihydroxyvitamin D_3.

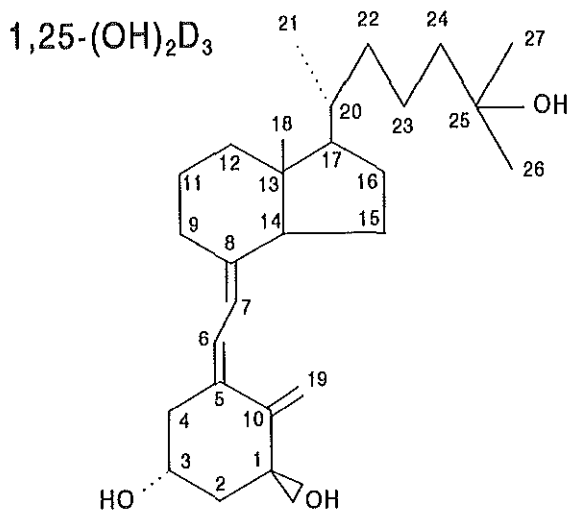

Figure 5

Chemical structure of the seco-steroid 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$).

1,25-$(OH)_2D_3$, providing a negative regulatory feedback loop in the synthesis of the biologically active form of vitamin D_3 (74-76).

Also in other target cells, 1,25-$(OH)_2D_3$ stimulates 24-hydroxylase enzyme activity, thereby initiating its own C-24 catabolic pathway. 24-Hydroxylase converts 1,25-$(OH)_2D_3$ to 1,24,25 trihydroxyvitamin D_3 (77).

Vitamin D_3 metabolites circulate in the blood primarily in equilibrium association with the vitamin D binding protein (DBP) (78), but also several other plasma proteins, including albumin, low density lipoprotein (LDL), high density lipoprotein (HDL), and chylomicrons (79,80), bind vitamin D_3 metabolites.

1.2.2 Mechanism of action of vitamin D

1,25-$(OH)_2D_3$ exerts its principal biological action through binding to the vitamin D receptor (VDR), although non-genomic actions of 1,25-$(OH)_2D_3$ have also been reported (81-84).

The VDR is a member of the steroid/thyroid hormone receptor superfamily that consists of intracellular transcription factors that, upon activation by their cognate ligand,

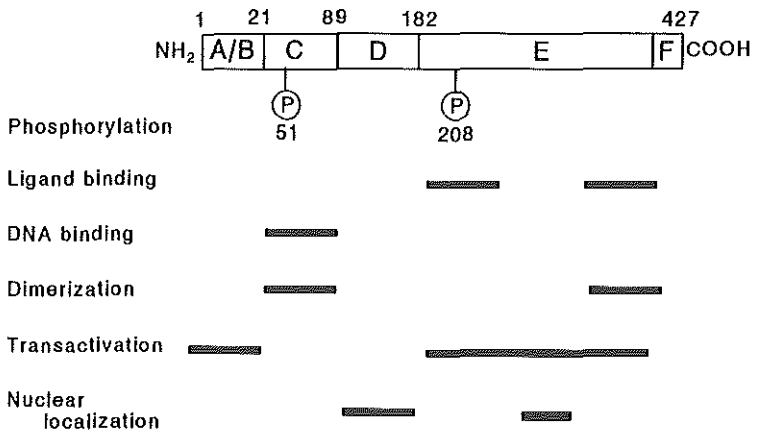

Figure 6

Vitamin D receptor structure, subdivided into regions with specific functions. Only phosphorylation sites for which functional importance has been reported, are indicated.

exert their action through binding to a DNA element (hormone response element), thereby modulating transcription of a *cis*-linked gene. The human VDR gene consists of 9 exons (85) and is located on chromosome 12q13-14 (86-88). The full length hVDR cDNA comprises 4,605 bp. The exact start site of the open reading frame is still under debate. Initially, the hVDR cDNA has been defined as a 115 bp noncoding leader sequence, 1,281 bp open reading frame and 3,209 bp of 3' noncoding sequence.

The VDR protein structure consists of 427 amino acids and can be subdivided into six regions (A to F) to which specific functions have been assigned including ligand binding, dimerization, nuclear localization, DNA binding and transactivation (Figure 6) (89-93). Among members of the steroid hormone receptor superfamily, the NH_2-terminal domain (A/B), which is highly variable in sequence and length, is important for the transactivation function. This region is involved in the activation of target gene transcription and probably achieves its effect by interacting with the core transcriptional machinery and/or other transactivating factors.

The DNA binding domain (DBD) (Figure 6, region C) exhibits the highest degree of homology among members of the steroid hormone receptor superfamily. This region represents a globular fold which contains two distinct zinc clusters, that consist of zinc ions that are tetrahedrally coordinated by four cysteine residues located at the base of each loop (Figure 7). The so-called P-Box is located in the first zinc cluster and contains

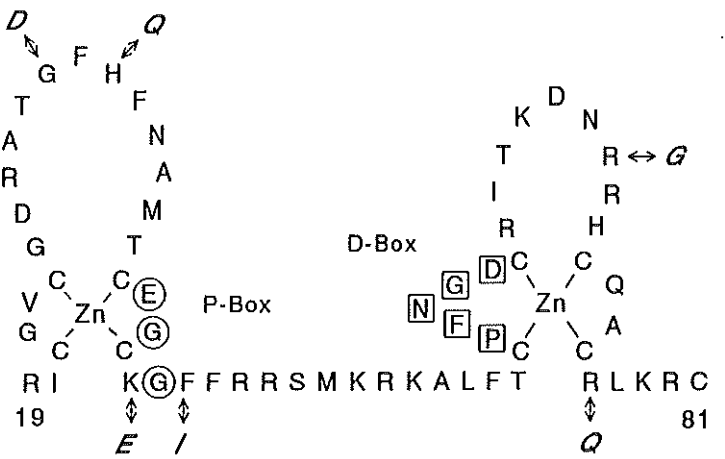

Figure 7

Part of the DNA binding domain of the vitamin D receptor, containing two distinct zinc (Zn) clusters. Circled amino acids belong to the P-Box, whereas boxed amino acids belong to the D-Box. Arrows indicate amino acids for which substitution mutations have been found in patients with hereditary vitamin D resistant rickets.

the second pair of zinc contacting cysteines. Three amino acids in the P-Box have been shown to be essential for recognition (cEGckG) of the vitamin D response element (VDRE). The Glu (E) residue has been shown to contact specifically the third nucleotide in the Vitamin D response element (94) (Figure 7). The α-helical structure of the P-Box and downstream amino acids is important for deoxynucleotide contacts and fits into the major groove of the DNA helix (95). The D-Box, that consists of the five amino acids between the NH_2-terminal pair of cysteines in the second zinc cluster, is important for contacts with the phosphate backbone of DNA and for dimerization. For dimerization also regions in the first Zn finger and in the T-region, which is localized COOH-terminal in the DBD close to the hinge region, have been shown to be important (96,95). Interaction of the aromatic side chains of conserved amino acids in the amphipathic helices provides the globular structure of the DBD. Importance of individual amino acids in the DBD for the functionality of the VDR is furthermore illustrated by mutations in the DBD that occur in hereditary vitamin D resistant rickets (HVDRR). Several mutations in the DBD have been identified in patients with HVDRR, including substitution mutations at the tip and at the base of the first Zn finger, amino acids 30 (85), 32 (98) and 42, 44 (99), respectively, as well as the second Zn finger, amino acids 70 (85) and 77 (100,101), respectively (Figure 7). Furthermore, a mutation has been identified which introduced a stop codon at codon 70 in the DBD, leading to the formation of a truncated VDR (102).

Downstream of the DBD is the hinge region, which is involved in nuclear localization (Figure 6, region D). In some patients with HVDRR a mutation, introducing a stop codon at codon 149, has been identified in the hinge region, which leads to the formation of a truncated VDR (102,103). The ligand-binding domain is located COOH-terminally of the hinge region (Figure 6, region E). Residues involved in ligand binding reside at either end of the ligand binding domain. Furthermore, the ligand binding domain is involved in heterodimerization and in protein-protein interactions leading to transcriptional activation as well as nuclear localization (104,105). A mutation in the ligand binding domain of the VDR has been identified at codon 271 in patients with HVDRR leading to absence of a biological response to 1,25-$(OH)_2D_3$ (103). Like in DBD and hinge region, also in the ligand binding domain a mutation has been found that introduces a stop codon (codon 292), leading to the formation of a truncated VDR (102,106). For the small COOH-terminal region (Figure 6, region F) no specific function has been identified yet.

Binding of 1,25-$(OH)_2D_3$ alters the conformation of the VDR and enhances the phophorylation of the VDR. The VDR can be phosphorylated at several sites (107) by several kinases, including protein kinase C (108,109), cAMP-dependent protein kinase A (110) and casein kinase II (111,112). Specific phosphorylation sites are located at serine residues 51 (108) and 208 (112). Phosphorylation of the VDR is important for its binding to the vitamin D responsive elements (discussed below) and for its transactivation function (113). The unoccupied VDR is predominantly localized in the cytoplasm (114). Upon binding of its ligand, the cytosolic VDR is transported to the nucleus through a microtubule-mediated mechanism (115,116). The importance of translocation of the VDR to the nucleus for the 1,25-$(OH)_2D_3$ bioactivity is illustrated by a case of HVDRR, where translocation to the nucleus was impaired (117).

To regulate transcriptional activity, the activated nuclear VDR binds to specific nucleotide sequences in the promoter regions of vitamin D responsive genes. These so-called vitamin D response elements (VDREs) consist of direct repeats of hexameric half elements spaced by three nucleotides. For high affinity binding to the VDRE the VDR requires a binding partner, termed receptor auxiliary factor (RAF) or nuclear accessory factor (NAF) (118-121). Isoforms of the retinoic-X-receptor (RXR) have been identified as the possible receptor auxiliary factor. In vitro synthesized RXR forms heterodimers with VDR and binds to the VDRE (122-124). The porcine intestinal accessory factor has recently been shown to be a RXR (125). In human osteoblastic cells, the endogenous accessory factor has been shown not to be RXR alpha, despite of the ability of RXR

alpha and VDR, synthesized in vitro, to bind the hOC VDRE (126). Three RXR genes have been identified (127), which suggests that various VDR-RXR heterodimers may control transcription differently or possibly affect transcription of different genes. Recently, interaction of the VDR with a component of the core transcriptional machinery, TFIIB, has been shown. The C-terminal ligand binding domain of the VDR interacts with a 43-residue amino-terminal domain of TFIIB (128,129). Whether the nuclear protein complex binding to the VDRE contains additional proteins or factors is unknown. In contrast to heterodimers, several groups have suggested the binding of VDR/VDR homodimers to certain VDREs (130-133).

Distinct VDREs, which have the potential to interact with different receptor homo- and heterodimers have been identified in the promoters of several genes, including the human (43,44) and rat (30-32) osteocalcin, mouse osteopontin (73), rat cytochrome P450 24-hydroxylase (132,134-136) as well as the mouse calbindin D28k (137), chicken ß integrin (138) and chicken carbonic anhydrase-II genes (139) (Table 2). In the human PTH gene a VDRE has been identified that consists of a single hexameric element and mediates transcriptional repression in response to 1,25-$(OH)_2D_3$ (140).

Members of the steroid/thyroid hormone receptor family bind HREs with distinct spacing requirements between the half sites of the HREs (141,142). Within the VDREs, however, also differences have been found in spacing between half sites and the identity of the complexes capable of binding to VDREs (130-133). VDR/VDR homodimers bind to hexanucleotide repeats with 6 nucleotides spacing, whereas VDR/RXR heterodimers bind to VDREs with three nucleotides spacing. However, conflicting results have been

Table 2

Sequences of vitamin D response elements (VDREs) in the promoters of several genes.

rat osteocalcin VDRE	5'-GGGTGA atg AGGACA-3'
human osteocalcin VDRE	5'-GGGTGA acg GGGGCA-3'
mouse osteopontin VDRE	5'-GGTTCA cga GGTTCA-3'
rat cytochrome $P450_{24}$ VDRE	5'-AGGTGA gtg AGGGCG-3'
rat calbindin D-28K VDRE	5'-GGGGGA tgt GAGGAG-3'
avian integrin $ß_3$ VDRE	5'-GAGGCA gaa GGGAGA-3'
chicken carbonic anhydrase II VDRE	5'-GGGGGA aaa AGTCCA-3'

Table 3

Sequences of vitamin D response elements (VDREs) for which VDR/VDR homodimer binding has been reported.

human osteocalcin VDRE	5'-GGGTGA ctcacc GGGTGA-3'
rat cytochrome $P450_{24}$ VDRE	5'-GGTCGA gcccag GGTTCA-3'
mouse osteopontin VDRE	5'-GGTTCA cga GGTTCA-3'

obtained in determining the character of the dimers that bind the osteopontin VDRE, which is a VDRE with 3 nucleotides spacing between the half elements. Carlberg et al. (130) showed that RXR is required for VDR binding to the osteopontin VDRE, whereas Freedman et al. (131) and Nishikawa et al. (133) reported VDR/VDR homodimer binding to the osteopontin VDRE (Table 3).

Ligand binding seems to be a prerequisite for binding of the VDR complex to the VDRE, especially for binding of heterodimers to the VDRE (143). Also ligand-independent VDR-DNA interaction has been described (121). In the presence of the ligand for RXR, 9-cis retinoic acid, heterodimer binding to the VDRE is reduced (124,144).

Finally, interaction of nuclear protein complexes with regulatory elements depends on chromatin structure. Genes located in heterochromatin are unable to bind transcription factors. In euchromatin regions the expression of genes also depends on chromatin structure. Positioning of nucleosomes determines the availability of regulatory elements. Furthermore, nucleosomes may play an important role in the interaction between regulatory elements and/or regulatory elements and the basal transcriptional machinery (145,146).

Figure 8 shows a model for the genomic mechanism of action of 1,25-$(OH)_2D_3$ in target cells.

1.2.3 Effects of Vitamin D

The main effect of 1,25-$(OH)_2D_3$ is elevation of plasma calcium levels in order to maintain plasma calcium homeostasis. Therefore, 1,25-$(OH)_2D_3$ exerts effects on the intestine, kidney and bone. In response to slight hypocalcemia PTH secretion is increased, followed by an increase in renal 1,25-$(OH)_2D_3$ production, which stimulates transepithelial calcium transport across the gut. Both PTH and 1,25-$(OH)_2D_3$ increase calcium transport from the renal tubular lumen into the bloodstream. In addition, 1,25-

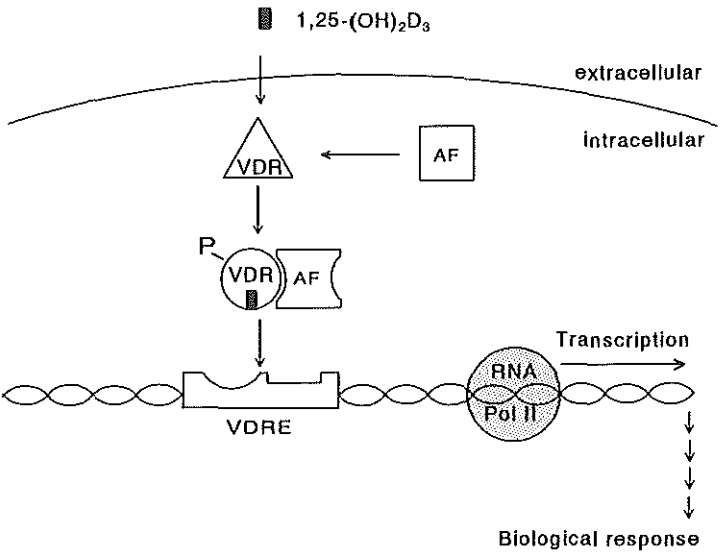

Figure 8

Genomic mechanism of action of 1,25-dihydroxyvitamin D_3. VDR = vitamin D receptor, AF=accessory factor, P = phosphorylation, VDRE = vitamin D response element, RNA pol II = RNA polymerase II

$(OH)_2D_3$ can increase PTH-stimulated bone calcium mobilization. Once calcium needs are met, PTH secretion is suppressed as well as production of 1,25-$(OH)_2D_3$ (147,148).

1,25-$(OH)_2D_3$ affects skeletal metabolism by regulating proliferation and differentiation of osteoblasts (3) and several parameters of osteoblastic function, including VDR level (149), alkaline phosphatase activity (150), and the production of several proteins (151): osteocalcin (15-17), osteopontin (64) and procollagen type I (152), IGF-I and IGF-BPs (153,154), IGF receptors (155), and TGFß (156,157). Furthermore, 1,25-$(OH)_2D_3$ affects cytoskeletal proteins (158,159). Previously, 1,25-$(OH)_2D_3$ effects on the osteoclast were thought to be exerted indirectly via the osteoblasts (160). However, recently, VDR transcripts have been shown also in osteoclasts, suggesting that 1,25-$(OH)_2D_3$ may modulate osteoclast function directly (161). Furthermore, 1,25-$(OH)_2D_3$ modulates differentiation of osteoclast progenitors (162).

Besides the effects of 1,25-$(OH)_2D_3$ on classical target tissues, 1,25-$(OH)_2D_3$ has been shown to affect several non-classical target tissues, of which many have been reported to contain the VDR, including the hematolymphopoietic system, muscles, brain, mammary gland, placenta, pancreas, thyroid, lung, liver and skin (148). In addition to normal tissues and organs, VDRs have been identified also in several malignant cell

types. Inhibition of proliferation of cancer cells by 1,25-$(OH)_2D_3$ has led to the speculation that 1,25-$(OH)_2D_3$ may be clinically useful as an anticancer agent (163-166).

1.3 LOCALLY PRODUCED FACTORS IN BONE

1.3.1 Introduction

A large series of growth factors and cytokines is produced by bone cells or in the vicinity of bone, e.g. bone marrow cells. These locally produced factors can broadly be subdivided into bone matrix bound and soluble factors (Table 4). Among them are transforming growth factor ß (TGFß) type 1 and 2, insulin-like growth factors I (IGF-I),

Table 4

Matrix bound and soluble factors locally produced in bone

Matrix bound factors
Transforming growth factor ß (TGFß)
Bone morphogenetic proteins (BMPs)
Insulin-like growth factors (IGF-I, IGF-II)
Platelet derived growth factor (PDGF)
Fibroblast growth factors (FGFs)
Other matrix proteins, including osteocalcin, osteopontin, bone sialo protein (BSP) (see also Table 2)
Soluble factors
Interleukins (IL-1, IL-6, IL-10)
Prostaglandins (PGs)
$ß_2$ Microglobulin
Tumor necrosis factors (TNFα, TNFß)
gamma-Interferon (gamma-IFN)
Macrophage colony-stimulating factor (M-CSF)
Granulocyte macrophage colony-stimulating factor (GM-CSF)
Leukemia inhibitory factor (LIF)

and bone morphogenetic protein-2 (BMP-2), which have been investigated in this study.

1.3.2 The TGFß superfamily

TGFß and BMP-2 belong to the TGFß superfamily. The TGFß superfamily is divided in several subfamilies, which show a higher or lower amino acid sequence homology. Seven cysteine residues are highly conserved in members of the superfamily, which is subdivided into the TGFß, Müllerian inhibitory substance (MIS), inhibin/activin, and the Vg-related subfamilies. Bone morphogenetic proteins 2 belongs to the Vg-related subfamily (Figure 9).

	MIS	Inhibin β A, B	Inhibin α	TGF-$\beta_{1,2}$	Vg1	DPP	BMP-5-7	BMP-3
BMP-2, 4	26-28	40-41	20-22	33-35	51-53	68-72	53-57	43-45
BMP-3	30	36-37	27	32	44	41	37-43	
BMP-5-7	23-26	35-44	22-23	33-37	51-53	50-53		
DPP	25	38-40	22	32-34	45			
Vg1	31	36-46	24	36				
TGF-$\beta_{1,2}$	24-28	35-42	24-26					
Inhibin α	25	26						
Inhibin β A, B	23-24							

Figure 9

Comparison of the amino acid sequences of the mature regions of TGFß superfamily members as reported by Rosen and Thies (227). Percentage of amino acid homology is depicted.

1.4 TRANSFORMING GROWTH FACTOR ß

1.4.1 TGFß structure

TGFß is synthesized and secreted in a latent, biologically inactive form (167,168), and consists of the two polypeptide chains noncovalently linked to the remainder of the TGFß proregion. This proregion prevents interaction of TGFß with TGFß cell surface receptors (169) (Figure 10). In addition, a TGFß binding protein, which varies in size depending on the cell type by which it is expressed, is disulphide linked to the TGFß proregion of the latent complex (170). In serum, an additional latent form of TGFß exists

consisting of TGFß complexed with α_2 macroglobulin (171). TGFß can be activated by extreme pH conditions, e.g. by the low pH under the ruffled border of the osteoclast during bone resorption (172-174) or by proteolytic activity (175) (Figure 10). However, activation of latent TGFß has also been reported to occur through binding to the IGF-II receptor (176). In mammalian cells three different types of TGFßs have been identified, $TGF\beta_1$, $TGF\beta_2$ and $TGF\beta_3$. In addition, $TGF\beta_4$ and $TGF\beta_5$ have been identified in the

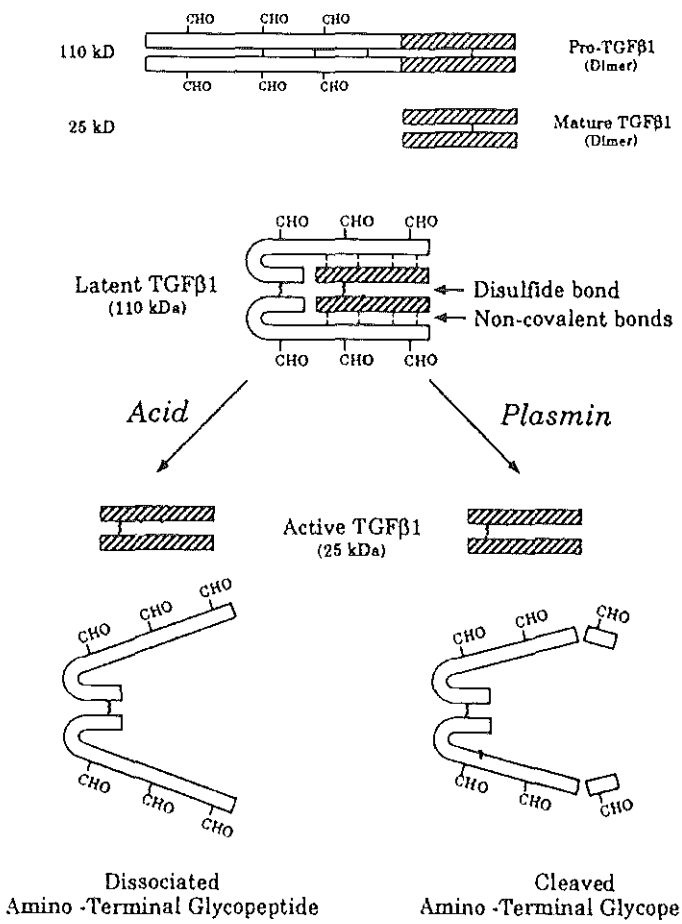

Figure 10

Structure of TGFß and activation of latent TGFß by low pH and plasmin as reported by Daopin et al. and Lyons et al. (97,173).

chicken and Xenopus laevis genomes, respectively. Besides homodimers, also TGFß heterodimers consisting of one polypeptide chain from one type of TGFß linked to a polypeptide chain from anothert type of TGFß, e.g. TGFß1.2 and TGFß2.3, have been identified (177).

1.4.2 Expression of TGFß

TGFß is expressed by a number of cell types, both normal and oncogenically transformed cells (178). In particular, TGFß is expressed during embryonic development, when expression of TGFß occurs in a spatial and temporal pattern, particularly in tissues of mesenchymal or mesodermal origin. High expression is observed at sites undergoing intense development and morphogenesis, including osteogenesis, (179,180). The largest amounts of stored TGFß in the body are found in bone, although platelets contain the highest concentrations of TGFß.

1.4.3 Mechanism of action of TGFß

TGFß binds to three cell surface receptors, two glycoproteins of 53 and 70-100 kD, called receptors I and II, respectively, and a membrane proteoglycan, designated betaglycan (181). Betaglycan may act as a component presenting TGFß to its signalling receptors I and II (182). TGFß receptors I and II are transmembrane receptors that contain a serine/threonine kinase domain on the cytoplasmic surface and are usually coexpressed in cells. The type I receptor is unable to bind ligand in the absence of type II receptors (183,184). Similarly, type II receptors are unable to signal in the absence of type I receptors (185). Type II receptors can interact with different type I receptor isoforms. The process of receptor activation is schematically depicted in Figure 11. The type II receptor is constitutively autophosphorylated and binds ligand independently of receptor type I. Subsequently, receptor I recognizes the TGFß/receptor type II complex and forms a stable complex with receptor type II. Until now, it is unknown whether receptor type I recognizes TGFß, which is conformationally changed after binding to receptor type II or that receptor type I recognizes the interface of the TGFß/receptor II complex. Next, receptor type I is phosphorylated on serine and threonine residues in the so-called GS domain of receptor I, a stretch of 30 amino acids preceding the kinase domain and conserved in type I receptors, by the kinase activity of receptor type II. Phosphorylation of the type I receptor is required for signal transmission. (186-188). Upon activation of the type I receptor a cascade of events may occur eventually leading to a biological response. Factors that have been suggested to belong to the signal

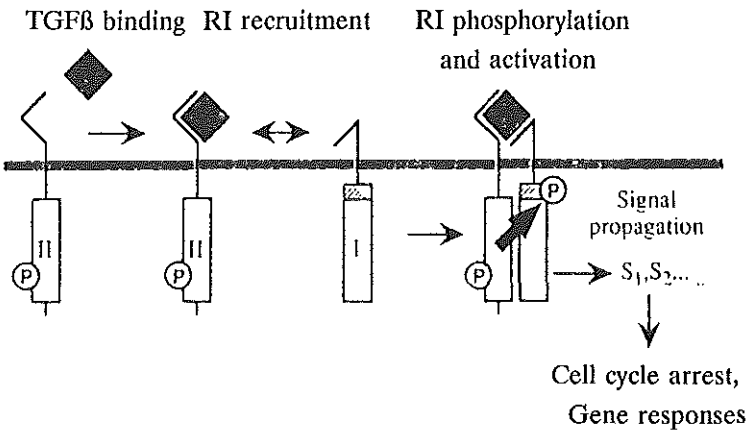

Figure 11

Mechanism of activation of TGFß type I and II receptors as reported by Wrana et al. (186).

transduction pathway of TGFß include protein kinase C (189-191), phosphatidylcholine-phospholipase C (192), myelin basic protein (MBP) kinases (193), Src family of protein tyrosine kinases (194) and p21ras (195,196). Recently, TGFß responsive elements have been identified in the osteocalcin gene (35) and the epidermal growth factor receptor gene (197). TGFß responsiveness of the osteocalcin gene involves binding of AP-1 proteins (35).

1.4.4 Effects of TGFß on bone

TGFß is involved in the process of bone formation and healing through stimulation of chondrogenesis and osteogenesis. Increased bone matrix apposition is observed after TGFß treatment and autoinduction of TGFß synthesis is observed in osteoblasts and chondrocytes (198-201). Stimulation of bone formation by TGFß is at least in part mediated through prostaglandin synthesis (202). TGFß has potent effects on bone cells in vitro. However, these effects depend on the maturation stage of the cells. In some cultured osteosarcoma cell lines, including ROS 17/2.8, UMR 106 and MG 63 cells, TGFß inhibits proliferation and stimulates differentiation (203,204), whereas in primary osteoblasts, MC3T3 and Saos cells TGFß stimulates proliferation and inhibits differentiation (203,205-209). Furthermore, TGFß affects the production of bone matrix proteins and other phenotypic markers of osteoblasts (26,67,204,210). Beside its effects on bone formation, TGFß also affects bone resorption. In the neonatal mouse organ culture system large doses of TGFß stimulate bone resorption (211). However, in fetal

rat long bone cultures bone resorption has been reported to be inhibited by TGFß (212). TGFß does not affect osteoclastic bone resorption directly (213), but primarily affects osteoclast precursor proliferation, fusion of precursors to form multinucleated osteoclasts and migration of osteoclasts (202,214-216). Furthermore, TGFß regulates matrix degradation by decreasing plasminogen activator activity and increasing the activity of plasminogen activator inhibitor (217-219). In transgenic mice, overexpression of $TGF\beta_1$ induced several skeletal abnormalities, including impaired bone mineralization and disrupted architecture of the epiphyseal growth plate (220).

1.5 BONE MORPHOGENETIC PROTEIN-2

1.5.1 Structure, expression and effects on bone

Nine BMPs have been identified, but only BMP-2 to BMP-9 are members of the TGFß superfamily. In Table 5 BMP-2 to BMP-9 are listed with their synonyms. BMPs are synthesized as dimeric precursor proteins that are posttranslationally processed into active 30 kD homodimeric proteins before secretion. They belong to the Vg-related subfamily of the TGFß superfamily.

BMP-2 has been localized in several embryonic organ systems, including heart,

Table 5

List of bone morphogenetic proteins belonging to the TGFß superfamily and their synonyms. BMP-1 is not a member of this family.

Bone morphogenetic	proteins (BMPs)
Factor	Synonym
BMP-2	BMP-2A
BMP-3	Osteogenin
BMP-4	BMP-2B
BMP-5	
BMP-6	
BMP-7	Osteogenic protein-1 (OP-1)
BMP-8	Osteogenic protein-2 (OP-2)
BMP-9	

nervous system, hair and tooth buds, as well as in areas of skeletal formation (221,222) and in osteosarcoma cells (223).

BMPs bind to a specific family of type I and II receptors related to the serine/threonine kinase receptors of the TGFß family, that are probably activated by a mechanism comparable to that of the TGFß family (224,225) (Figure 10).

BMP-2 is an osteoinductive factor that is, in contrast to TGFß, capable of inducing ectopic bone formation (226,227). In addition, BMP-2 has been shown to be involved in bone defect repair by inducing new bone formation (228,229). Moreover, the BMP-induced bone formation follows the sequence of events observed in embryonic endochondral bone formation: mesenchymal cells are transformed into chondroblasts, cartilage is formed and subsequently replaced by bone.

BMP-2 affects differentiation of mesenchymal stem cells into adipocytes, chondrocytes or osteoblasts depending on its concentration (230,231). BMP-2 has been shown to regulate proliferation and differentiation of embryonic skeletal precursor cells (232). *In vitro*, BMP-2 induces osteoblast differentiation of isolated cells from bone marrow and calvaria and of cell lines as indicated by increased expression of osteocalcin, collagen type I and alkaline phosphatase activity (233-239). Furthermore, BMP-2 enhances the expression of interleukin-6, TGFß 1 (240), IGF-I and IGF-II (241) in osteoblast-like cells.

1.6 INSULIN-LIKE GROWTH FACTOR-I

1.6.1 Structure, expression and effects on bone

IGF-I is a single-chain polypeptide with structural homology to proinsulin (48%). In serum, IGF-I is bound to an acid labile subunit and members of the family of the IGF binding proteins (IGFBPs). These act as transport proteins, prolong the half-life of IGFs, provide a means of tissue-specific localization and modulate interaction of IGFs with their receptors (242). Six IGFBPs have been cloned, of which IGFBP-1, IGFBP-3 and IGFBP-4 bind IGF-I. Except for IGFBP-1, all IGFBPs are produced in bone (243).

The IGF-I receptor mediates most cellular effects of IGF-I. This receptor is a membrane receptor that consists of an extracellular α-subunit linked to a transmembrane/cytoplasmic ß-subunit, which together form the αß half-receptor. The mature IGF-I receptor is formed by linkage of two half-receptors. The cytoplasmic part of the receptor contains tyrosine kinase activity and tyrosine residues that are autophosphorylated by the kinase of the other ß subunit upon ligand binding. Subsequently, insulin receptor substrate I (IRS-I) is phosphorylated, followed by a

cascade of events that has not yet been fully established (244).

IGF-I is expressed by a wide variety of cells and organs, including liver, testis, ovary, lymphoid and hematopoietic organs, salivary gland, adrenal medulla, parathyroid gland, nervous system, bone marrow and chondrocytes (245). Expression of IGF-I has also been reported in murine osteoblasts (246,247). However, conflicting results have been obtained on the expression of IGF-I in human osteoblasts (248,249). Receptors for IGF-I have been identified in fetal rat osteoblasts (250).

At the moment IGF-I is considered as an autocrine and paracrine stimulator of bone formation during bone remodelling. Systemic IGF-I may play a role in achieving normal longitudinal skeletal growth. IGF-I has been shown to enhance both osteoblast and osteoclast differentiation. IGF-I enhances procollagen type I mRNA levels and type I collagen synthesis in osteoblast-enriched cultures of fetal rat calvaria. At the same time, IGF-I decreases collagenase expression, thereby decreasing collagen degradation (251-253). Furthermore, IGF-I increases alkaline phosphatase activity and osteocalcin production (28,254-256). Besides the effects on the osteoblast, IGF-I also affects the osteoclast. Osteoclast formation and activity has been reported to be enhanced by IGF-I, although this effect may be mediated indirectly through the osteoblast (257,258).

1.7 SCOPE OF THE THESIS

Bone remodelling is a complex process, which needs to be tightly regulated. In this regulation both hormones and locally produced factors play a role. During bone formation, systemic factors may act either directly or indirectly on the osteoblast. Systemic factors may affect the osteoblast indirectly by modifying the synthesis, activity or binding of local factors (259). Local factors are synthesized by osteoblasts and nonosteoblastic skeletal cells and may act in an autocrine or paracrine way. In addition, local factors that are synthesized by the osteoblast during bone formation may act on the osteoclast, thereby linking bone formation to bone resorption. During bone resorption, osteoclasts resorb bone matrix and may thereby release factors that in turn act on the osteoblast. In some tissues local factors have been described to influence the synthesis of steroids or production of enzymes involved in the steroidogenesis (259).

In vitro, 1,25-$(OH)_2D_3$ has been shown to increase TGFß synthesis in osteoblasts (156) and *in vivo*, vitamin D deficiency resulted in a reduction of TGFß in bone (157). IGF-I release is inhibited by 1,25-$(OH)_2D_3$ in mouse osteoblastic cells (153,154).

Several reports have indicated the importance of local factors in the action of steroids on bone. Those reports primarily focussed on the effects of steroids on the

synthesis of local factors, which in turn affect the bone remodelling process. In contrast, the studies described in this thesis deal with the effect of the presence of local factors on the action of the systemic hormone, 1,25-dihydroxyvitamin D_3 in bone. The locally produced factors that were studied in this thesis are TGFß, BMP-2 and IGF-I, which have been identified as stimulators of bone formation.

The following topics have been studied: In Chapter 2 we investigated the effect of TGFß on VDR levels in osteoblast-like cells and studied the importance of receptor regulation for two biological responses to 1,25-$(OH)_2D_3$, osteocalcin and osteopontin expression. Next, we investigated the mechanism whereby TGFß modulates 1,25-$(OH)_2D_3$ enhancement of osteocalcin and osteopontin expression in osteoblast-like cells (Chapter 3). The importance of modulation of VDR level by TGFß for another biological response to 1,25-$(OH)_2D_3$, induction of 24-hydroxylase activity in osteoblast-like cells, is studied and described in Chapter 4.

Furthermore, we compared the effects of TGFß, BMP-2 and IGF-I on the 1,25-$(OH)_2D_3$-induced increase in osteocalcin and osteopontin expression (Chapter 5) and 1,25-$(OH)_2D_3$-stimulated bone resorption (Chapter 6). In Chapter 6, also the presence and distribution of these locally produced factors in bone was studied.

Finally, Chapter 7 deals with the identity of nuclear protein complexes that bind to the osteocalcin and osteopontin VDREs in the promoter regions of these genes. The results are discussed in Chapter 8 and summarized in Chapter 9.

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CHAPTER 2

TRANSFORMING GROWTH FACTOR ß-INDUCED DISSOCIATION BETWEEN VITAMIN D RECEPTOR LEVEL AND 1,25-DIHYDROXYVITAMIN D_3 ACTION IN OSTEOBLAST-LIKE CELLS

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Bone and Mineral 1994 volume 26, 27-42

ABSTRACT

In the present study the interaction between a locally produced factor in bone, transforming growth factor ß (TGFß) and a systemic regulator of bone metabolism, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) was investigated.

In rat (UMR 106, ROS 17/2.8) and human (MG 63) osteoblastic cell lines and in isolated fetal rat osteoblasts, TGFß caused a comparable increase in vitamin D receptor (VDR) level. A maximum was observed after 6 h at 1 ng/ml TGFß. Scatchard analysis revealed that up-regulation of VDR is due to an increase in receptor number and not to a change in affinity. This was supported by Northern blot analysis, which showed a dose- and time-dependent increase in VDR mRNA by TGFß. To assess the significance of the TGFß-induced increase in VDR level for 1,25-$(OH)_2D_3$ effects, cells were *pre*incubated with TGFß for 4 h (causing a 2-3 fold increase of the VDR level) and subsequently incubated with 1,25-$(OH)_2D_3$ for 4 h and 24 h. TGFß preincubation potently inhibited subsequent 1,25-$(OH)_2D_3$ stimulation of osteocalcin production in both ROS 17/2.8 and MG 63 cells on protein as well as mRNA level. A similar inhibition by TGFß was observed on the 1,25-$(OH)_2D_3$-induced increase in osteopontin mRNA.

The current study demonstrates dissociation between regulation of VDR level and modulation of two 1,25-$(OH)_2D_3$ biological responses by TGFß in osteoblast-like cell lines of different origin. This dissociation shows that besides interaction at VDR level also at other levels in the cell interaction(s) between TGFß and 1,25-$(OH)_2D_3$ exist. Besides, these data emphasize the potential importance of the interplay of locally produced factors and systemic calciotrophic hormones in the regulation of bone metabolism.

INTRODUCTION

The seco-steroid 1,25-$(OH)_2D_3$ is an important regulator of calcium homeostasis. Besides stimulation of calcium absorption in the intestine an important effect of 1,25-$(OH)_2D_3$ in response to a decrease in serum calcium concentration is stimulation of bone resorption (1). The vitamin D receptor (VDR) in bone is located in the bone forming cell (osteoblast) and not in the bone resorbing cell (osteoclast) (2,3). This indicates that 1,25-$(OH)_2D_3$ stimulates bone resorption indirectly via the osteoblast (4).

Regulation of receptor number is thought to be an important mechanism by which target cell responsiveness to hormones is modulated. Several factors, including 1,25-$(OH)_2D_3$ itself (5,6), glucocorticoids (7,8), parathyroid hormone (9,10), epidermal growth factor (11) and retinoic acid (12), have been demonstrated to modulate VDR level. Osteoblast responsiveness to 1,25-$(OH)_2D_3$ has been linked to VDR level. Induction of 24-hydroxylase activity by 1,25-$(OH)_2D_3$ closely paralleled VDR level after retinoic acid pretreatment (12). For both a stimulatory (24-hydroxylase) and inhibitory (collagen synthesis) bioresponse (13) the magnitude of the response to 1,25-$(OH)_2D_3$ was directly correlated with the abundance of receptors (depending on rate of cell proliferation). Chen et al. (8,14) showed that receptor up-regulation increased the magnitude and sensitivity of responses to 1,25-$(OH)_2D_3$. However, with another steroid hormone, glucocorticoid, a dissociation was observed between receptor regulation and bioresponse (15).

Transforming growth factor ß (TGFß) is produced in bone, and present as a latent complex (16). TGFß has been thought to be activated during bone resorption by the low pH under the ruffled border of the osteoclast and to play an important role in the local regulation of bone remodeling (16,17). TGFß has been shown to increase VDR level in UMR 106-06 cells after 3 days of incubation (18). Interaction between 1,25-$(OH)_2D_3$ and TGFß has been described in MG 63 cells in the regulation of biological responses after coincubation (19).

In the present study we investigated the effect of TGFß on VDR level in several osteoblast-like cell lines and studied the significance of this modulation of VDR level for 1,25-$(OH)_2D_3$ stimulation of osteocalcin production, osteocalcin mRNA and osteopontin mRNA synthesis. In general, this study emphasizes the importance of interactions between systemic and locally produced factors for the regulation of bone metabolism.

MATERIALS AND METHODS

Materials

[23,24-^{3}H]-1,25$(OH)_2D_3$ (105.5 Ci/mmol) was obtained from Amersham International (Aylesbury, Buckinghamshire, United Kingdom) and non-radioactive 1,25$(OH)_2D_3$ was generously provided by LEO Pharmaceuticals BV (Weesp, the Netherlands). TGFß type 2 was generously provided by Dr. J. Feyen, Sandoz Pharma Ltd (Basel, Switzerland). Fetal calf serum (FCS), αMEM medium, penicillin, streptomycin and glutamine were purchased from Flow Laboratories (Irvine, United Kingdom) and BSA fraction 5 from ICN Pharmaceuticals, Inc. (Costa Mesa, CA, USA). The rat VDR cDNA probe (1.7 kb) was generously provided by Dr. J.W. Pike (Houston, TX, USA), the rat osteocalcin probe (0.52 kb) and the rat osteopontin probe (1.3 kb) by Dr. M. Noda (West Point, PA, USA). Rat osteocalcin

antiserum was a generous gift of Prof. R. Bouillon (Leuven, Belgium). All other reagents were of the best grade commercially available.

Cell culture

MG 63 cells were generously provided by Prof. R. Bouillon and ROS 17/2.8 cells by Dr. S.B. Rodan (Merck, Sharp & Dohme Research Laboratories, West Point, PA, USA). UMR 106, ROS 17/2.8 (rat), and MG 63 (human) cells were seeded at 50,000 cells/cm^2 and cultured for 24 hours with αMEM supplemented with 2 mM glutamine, 0.1% glucose, 100 U/ml penicillin, 100 U/ml streptomycin and 10% fetal calf serum (FCS). Next, medium was replaced by αMEM with 2% charcoal-treated FCS and cells were cultured for a 24 h- or 48 h-period during which the cells reached confluence. During this period, cells were incubated with the agents to be tested. Fetal rat osteoblasts were isolated from 20-day old fetal calvaria by sequential EDTA and collagenase treatment (20), seeded at 25,000 cells/cm^2 and cultured for four days as described for the cell lines.

Preparation of cell extracts and [^{3}H]1,25(OH)$_2$D$_3$ binding assay

For single point assays, conditions were employed which were previously shown to provide valid estimates of total receptor content in cytosolic extracts (6). Cells were incubated and cultured as described above. Next, the cell pellet was extracted on ice in a hypertonic buffer consisting of 300 mM KCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM dithiothreitol, 10 mM sodium molybdate and 0.1 % Triton X-100. High-speed supernatants were obtained and 250 µl aliquots were incubated overnight at 4°C: 1) for single point assays with 0.25 nM [^{3}H]1,25-(OH)$_2$D$_3$ or 2) for scatchard analysis with several concentrations of [^{3}H]1,25-(OH)$_2$D$_3$ (ranging from 0.2 nM to 0.01 nM), in the absence or presence of a 200-fold molar excess of unlabeled hormone. Receptor bound 1,25-(OH)$_2$D$_3$ was separated from unbound ligand by charcoal adsorption (21).

Osteocalcin assay

The incubation protocol to study the significance of TGFß-induced increase in VDR level for the 1,25-(OH)$_2$D$_3$-stimulated osteocalcin production is shown in the inset in Fig. 5A. Cells were preincubated with TGFß for 4 h followed by the addition of 1,25-(OH)$_2$D$_3$ or vehicle without removal of TGFß. Osteocalcin measurement in medium of MG 63 cells was performed by use of the INCSTAR (Stillwater, Minnesota, USA) ^{125}I RIA kit for osteocalcin. Before measurements medium was freeze dried and reconstituted in assay buffer, so that medium was concentrated 6-fold. Cellular osteocalcin level of MG 63 cells was below detection limit. Osteocalcin measurements in cells and medium of ROS 17/2.8 cells were performed according to the method described by Verhaeghe et al. (22). Sample preparation of cell extracts: cells were washed in PBS-triton X-100 (0.1%), removed in 0.5 ml 6 M guanidine-HCl in 0.1 M Tris-buffer (pH 8.0) and next assayed for osteocalcin.

DNA and protein measurements

Changes in DNA content were assessed by the fluorimetrical method of Johnson-Wint and Hollis (23). Protein concentration was measured according to the method of Bradford (24).

Northern and dot blot analysis

RNA isolation was performed according to the method of Chomszinski (25). Electrophoresis of total cytoplasmic RNA (30 µg) through a formaldehyde gel and Northern blotting (Gene Screen filters)

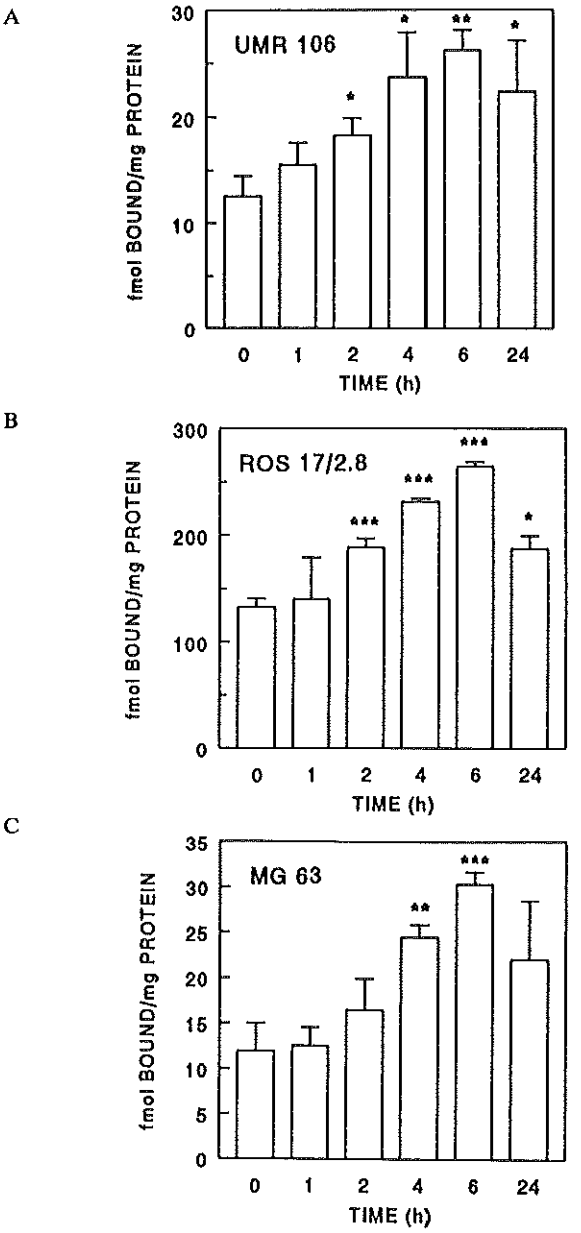

Figure 1

Time-course of the effect of 1 ng/ml TGFß on VDR level in A) UMR 106, B) ROS 17/2.8 and C) MG 63 cells. Cells were cultured as described in Materials and Methods and incubated for the time indicated with 1 ng/ml TGFß. Data are expressed as mean ± SD of single point assays in duplicate of two separate experiments. * $p<0.05$; ** $p<0.005$; *** $p<0.001$ versus control

were performed according to the method described by Davis et al. (26). Dot blots were performed according to the method described by Sambrook et al. (27).

Hybridization of Northern blots: Northern blots were prehybridized for 2 hours at 42°C in a buffer containing 50% formamide, 0.2% SDS, 1x Denhardt's solution, 5x SSC (1x SSC = 150 mM NaCl and 15 mM Na citrate, pH 7.0), 20 mM NaH_2PO_4 , 6% dextran sulphate, 1 μg/ml herring sperm DNA and then hybridized for 16-24 h at 42°C with ^{32}P-labeled cDNA probe. Filters were washed twice with 2x SSC and 0.5% SDS for 5 minutes at room temperature, once with 2x SSC and 0.5% SDS for 30 minutes at 50°C and once in 1x SSC and 0.5% SDS for 30 minutes at room temperature and exposed to X-ray films. The cDNA probes included 1.7 kb rat VDR fragment, 0.52 kb rat osteocalcin fragment, 1.3 kb rat osteopontin fragment, 1.2 kb hamster ß-actin fragment, and 0.8 kb human GAPDH fragment. For rehybridization filters were washed for 2 h at 65°C with 5 mM Tris-HCl (pH 8.0), 0.2 mM Na_2EDTA (pH 8.0), 0.05% sodiumpyrophosphate and 0.1x Denhardt's solution.

Data analysis

To assess significance of interaction between two agents tested data were analyzed with analysis of variance for 2-way design. Other statistical analyses were performed using Student's t-test.

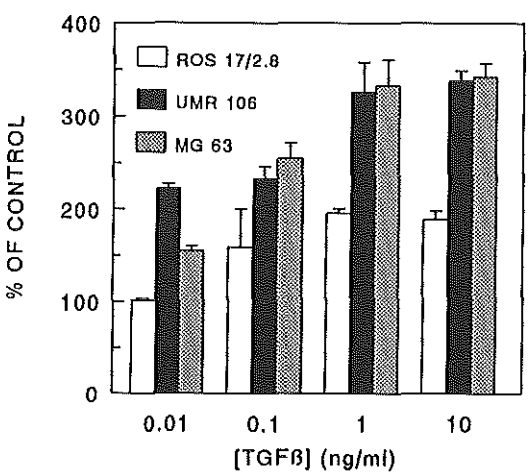

Figure 2

Responses to different doses of TGFß of VDR level in ROS 17/2.8 (open bars), UMR 106 (solid bars) and MG 63 (semi-solid bars) cells. Cells were cultured as described in Materials and Methods and incubated for 4 h with TGFß. Data are expressed as percentage of control of single point assays (fmol bound/mg protein) in duplicate of two separate experiments. Increases in VDR level are significant ($p<0.001$ versus control) in all incubations, except for ROS 17/2.8 cells at 0.01 ng/ml TGFß.

RESULTS

The effect of TGFß on VDR level in several osteoblast-like cells.

In UMR 106 (Fig. 1A), ROS 17/2.8 (Fig 1B) and MG 63 (Fig. 1C) cells 1 ng/ml TGFß caused a time-dependent increase in 1,25-$(OH)_2D_3$ binding with a maximum after 6 h. After 24 h 1,25-$(OH)_2D_3$ binding decreased towards prestimulation level in all cell lines, although 1,25-$(OH)_2D_3$ binding was still significantly elevated in both ROS 17/2.8 and UMR 106 cells. As shown in Fig. 2, in all three cell lines tested TGFß caused a dose-dependent up-regulation of the VDR. A maximum effect was observed with 1 ng/ml TGFß. In UMR 106 and MG 63 cells a significant increase was already observed. after treatment with 0.01 ng/ml TGFß, whereas in ROS 17/2.8 cells a first significant increase in VDR level was observed after incubation with 0.1 ng/ml TGFß. The ED_{50} for the effect on VDR was 0.05-0.1 ng/ml in UMR 106 and MG 63 cells and about 0.5 ng/ml in ROS 17/2.8.

In ROS 17/2.8 cells basal VDR level was higher than in MG 63 and UMR 106 cells. During the period we cultured the ROS 17/2.8 cells (about 20 passages) basal VDR decreased from 133.10 ± 8.22 fmol [^{3}H]1,25-$(OH)_2D_3$ bound/mg protein to 8.95 ± 0.49 fmol [^{3}H]1,25-$(OH)_2D_3$ bound/mg protein. However, independent of basal VDR level TGFß (1 ng/ml) caused a two-fold increase in VDR level (data not shown).

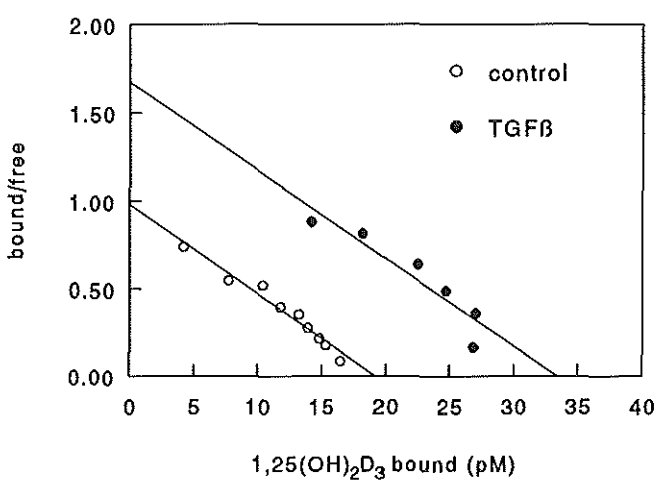

Figure 3

Scatchard analysis of TGFß effect on 1,25-$(OH)_2D_3$ binding in UMR 106 cells. Cells were cultured as described in Materials and Methods and incubated for 24 h with or without 1 ng/ml TGFß.

Besides these osteoblast-like cell lines also in isolated fetal rat osteoblasts TGFß (1 ng/ml) increased 1,25-$(OH)_2D_3$ binding (from 16.51 ± 0.31 fmol [^{3}H]1,25-$(OH)_2D_3$ bound/mg protein to 29.29 ± 1.28 fmol [^{3}H]1,25-$(OH)_2D_3$ bound/mg protein). Both for the cell lines tested and the primary cultures the maximal increase in VDR level at 1 ng/ml TGFß was 2-3 fold.

Scatchard analysis (Fig. 3) revealed that the increase in 1,25-$(OH)_2D_3$ binding in UMR 106 cells was not due to a change in the apparent dissociation constant of the VDR. An important role for new receptor synthesis in the TGFß effect on VDR is further demonstrated by mRNA analysis. TGFß increased VDR mRNA in ROS 17/2.8 (Figs. 4A and B) and UMR 106 cells (data not shown) in a dose- and time-

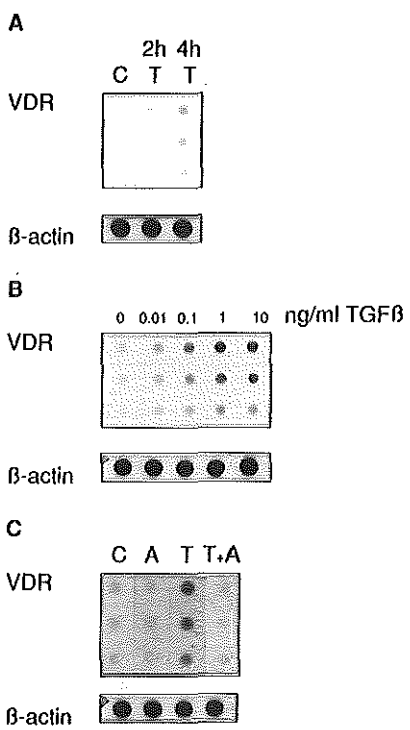

Figure 4

Effect of TGFß incubation on VDR mRNA in ROS 17/2.8 cells and the effect of coincubation with actinomycin D. Cells were cultured as described in Materials and Methods and incubated A) for 2 and 4 h with 1 ng/ml TGFß (T) or control (C) medium, or B) for 4 h with various doses of TGFß or C) for 4 h with or without 1 ng/ml TGFß (T) and 1 µg/ml actinomycin D (A). VDR mRNA data are shown in three dilutions of total RNA (15 µg, 5 µg and 1 µg). Actin mRNA reflects a representative of one of these dilutions.

dependent manner. The TGFß-induced increase in VDR mRNA in ROS 17/2.8 cells was completely blocked during coincubation with the transcription inhibitor actinomycin D (1 μg/ml) (Fig. 4C).

Effects of TGFβ pretreatment on 1,25-$(OH)_2D_3$ stimulation of osteocalcin production.

To study the relationship between receptor level and 1,25-$(OH)_2D_3$ effects on osteocalcin production cells (ROS 17/2.8 and MG 63) were *pre*incubated with TGFß for 4 h (causing an increase in VDR level) and subsequently incubated with 1,25-$(OH)_2D_3$ for 4 h and 24 h without removal of TGFß. The incubation protocol is shown in diagram in the inset in Fig. 5A. The effects of 4 h preincubation with three concentrations of TGFß (0.1, 1, 10 ng/ml) on subsequent stimulation (24 h) of osteocalcin production by various 1,25-$(OH)_2D_3$ concentrations (10^{-11}-10^{-8} M) were studied. Cell proliferation was not affected by the continuous presence (28 h) of TGFß in these experiments (data not shown). Basal osteocalcin production, measured in medium, was lower in MG 63 cells than in ROS 17/2.8 cells. In contrast to ROS 17/2.8 cells cellular osteocalcin content in MG 63 cells was below detection level. In both ROS 17/2.8 and MG 63 cells incubation with TGFß (4 h preincubation with TGFß followed by 24 h without addition of 1,25-$(OH)_2D_3$) did not modulate basal osteocalcin production (Figs. 5A and B). 1,25-$(OH)_2D_3$ caused a dose-dependent increase in osteocalcin production, which was not significantly affected by preincubation with 0.1 ng/ml TGFß in both ROS 17/2.8 (data not shown) and MG 63 (Fig. 5A) cells. However, 1 ng/ml (ROS 17/2.8 and MG 63 cells) and 10 ng/ml TGFß (ROS 17/2.8 cells), which doubled VDR level, inhibited the 1,25-$(OH)_2D_3$ stimulation of osteocalcin production (Figs. 5A and B). Studies with ROS 17/2.8 cells showed that 10 ng/ml TGFß was generally more potent than 1 ng/ml and that the effect of TGFß is not the result of inhibition of osteocalcin secretion as it could be observed both in the medium and cellular extracts (data not shown). Time course studies with ROS 17/2.8 cells revealed that an inhibitory effect of TGFß could be observed as soon as an effect of 1,25-$(OH)_2D_3$ on osteocalcin production was detected, i.e. in the cells after 8 h 1,25-$(OH)_2D_3$ incubation and in the medium after 16 h incubation (data not shown). The data on dissociation between TGFß-induced up-regulation of VDR and 1,25-$(OH)_2D_3$ stimulation of osteocalcin production in ROS 17/2.8 and MG 63 cells are illustrated in Figs. 6A and B for stimulation of osteocalcin production by 10^{-8} M 1,25-$(OH)_2D_3$. Next, we studied the effect of TGFß on the 1,25-$(OH)_2D_3$-induced increase in osteocalcin mRNA in ROS 17/2.8 cells. Basal osteocalcin mRNA was not affected after incubation with TGFß for 8 h (4 h TGFß preincubation followed by 4 h without 1,25-$(OH)_2D_3$) as well as after 28 h (4 h preincubation followed by 24 h without 1,25-$(OH)_2D_3$). Incubation for 24 h with 1,25-$(OH)_2D_3$ induced a significant

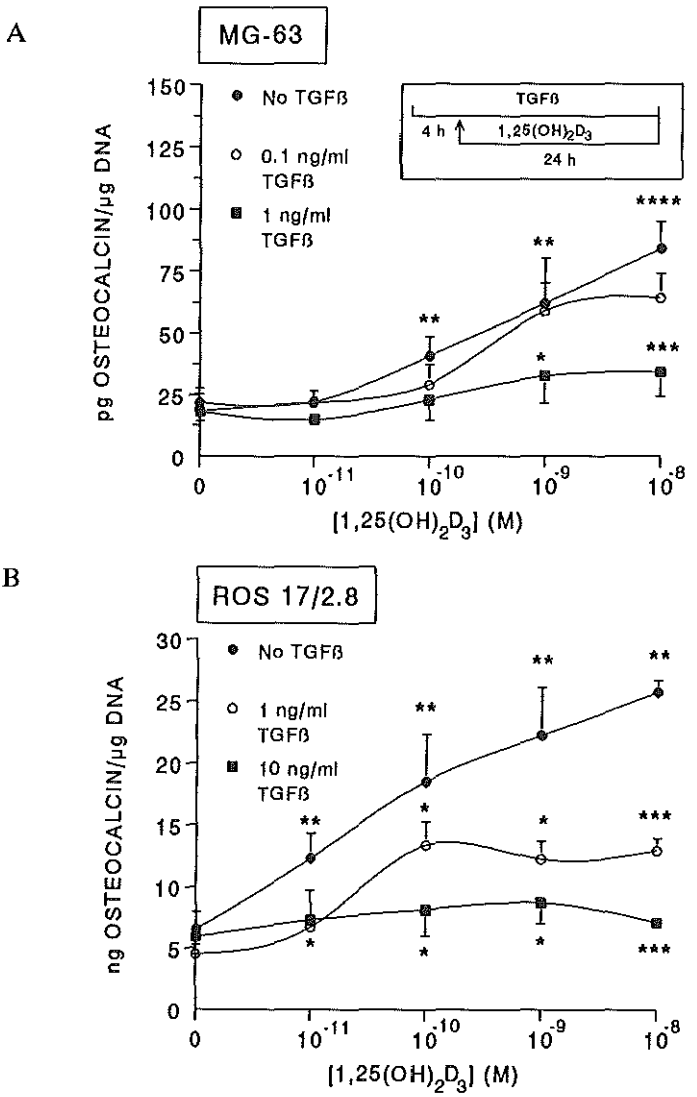

Figure 5

The effect of preincubation with TGFß on 1,25-$(OH)_2D_3$-induced increase in medium osteocalcin content of A) MG 63 and B) ROS 17/2.8 cells. Cells were cultured as described in Materials and Methods and incubated for 4 h with 0.1 or 1 ng/ml TGFß and subsequently (without medium change) for 24 h (as illustrated in the inset in Fig. 5A) with several doses of 1,25-$(OH)_2D_3$. Data are expressed as mean ± SD of single point assays in duplicate of two separate experiments. * $p<0.05$ calculated as significance of interaction, ** $p<0.005$ versus control, *** $p<0.005$ calculated as significance of interaction, **** $p<0.001$ versus control.

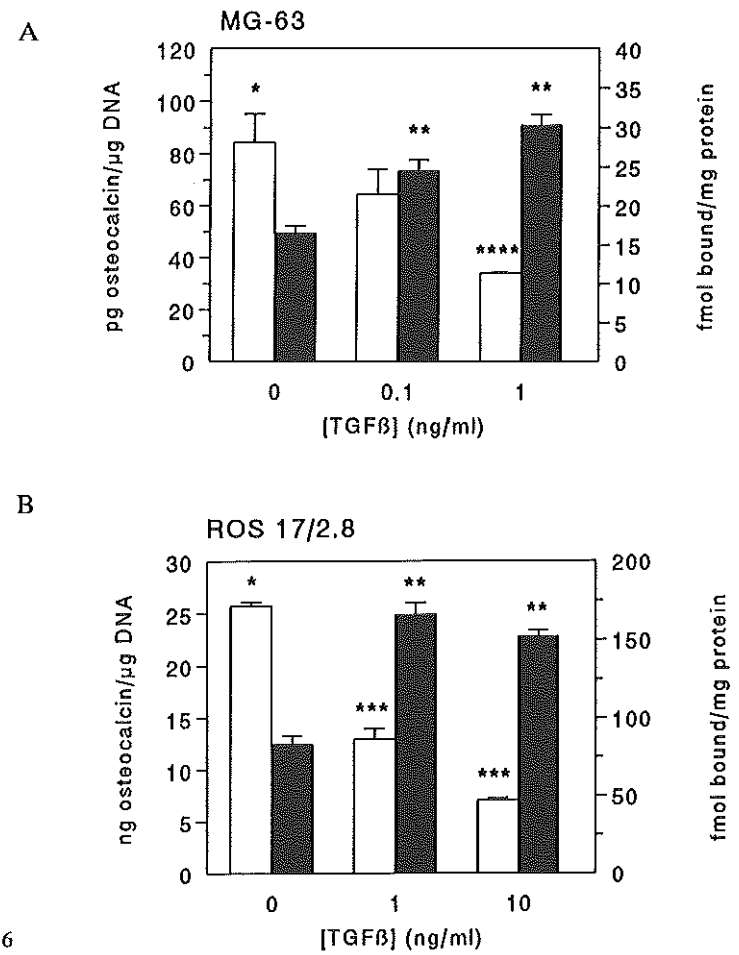

Figure 6

Effect of TGFß on VDR level in relation to its effect on 1,25-$(OH)_2D_3$-stimulated osteocalcin production in A) MG 63 and B) ROS 17/2.8 cells. Cells were cultured as described in Materials and Methods and incubated as shown in diagram in the inset of Fig. 5A. Data represent medium levels of osteocalcin (open bars) after 24 h incubation with 10 nM 1,25-$(OH)_2D_3$ with 4 h preincubation with TGFß and 1,25-$(OH)_2D_3$ binding data (solid bars) after 4 h of preincubation with TGFß expressed as mean ± SD of single point assays in duplicate of two separate experiments. * $p<0.005$, ** $p<0.001$ versus control and *** $p<0.001$, **** $p<0.005$ calculated as significance of interaction.

increase in osteocalcin mRNA, whereas incubation for 4 h with 1,25-$(OH)_2D_3$ induced a small, not-significant increase in osteocalcin mRNA (Fig. 7). Pretreatment for 4 h with 1 ng/ml TGFß almost completely blocked the increase in osteocalcin

mRNA by subsequent treatment with 1 nM 1,25-$(OH)_2D_3$ for 24 h. The 4 h 1,25-$(OH)_2D_3$ action was not significantly inhibited. However, there is certainly no potentiation of the 1,25-$(OH)_2D_3$ action as a result of the TGFß-induced up-regulation of the vitamin D receptor. Generally, data obtained on mRNA level confirm the effects observed on protein level.

Effect of TGFß pretreatment on 1,25-$(OH)_2D_3$ induction of osteopontin mRNA.

Similar experiments as described for osteocalcin measurements were performed to study the effect of TGFß pretreatment on 1,25-$(OH)_2D_3$ induction of osteopontin mRNA in ROS 17/2.8 cells. As shown in Fig. 8, 24 h 1 nM 1,25-$(OH)_2D_3$ induced an increase in osteopontin mRNA. After 28 h of incubation with TGFß (4 h preincubation with TGFß followed by 24 h without 1,25-$(OH)_2D_3$) a small, significant increase in basal osteopontin mRNA was observed. The 1,25-$(OH)_2D_3$-induced increase (24 h) was almost completely blocked by 4 h pretreatment with 1 ng/ml TGFß. In short term incubations (4 h TGFß followed by 4 h 1,25-$(OH)_2D_3$ incubation) both TGFß and 1,25-$(OH)_2D_3$ did not affect osteopontin mRNA synthesis significantly (Fig. 8).

Together, as for the data on osteocalcin, the data obtained with respect to osteopontin after 24 h demonstrated a dissociation between TGFß-induced up-regulation of VDR and the regulation of 1,25-$(OH)_2D_3$ biological activity (i.e. increased VDR level is not followed by a potentiation of 1,25-$(OH)_2D_3$ effects).

DISCUSSION

VDR levels have been described to be regulated by several factors (5-12,28), including TGFß (18). In contrast to the data of Schneider et al. (18), who observed an increase after 72 h, we already observed a TGFß effect on VDR level after 2 h with a maximum after 6 h. Our observations seem to represent a direct regulation of VDR level, whereas the data obtained by Schneider et al. may be the indirect result of changes in proliferation and differentiation of the cells. This is conceivable because TGFß modulates the proliferation and differentiation of osteoblasts (29-33) and the VDR level has been reported to vary between developmental stages of cells (34,35). However, in our experiments 28 h incubation with TGFß did not affect proliferation. Our data show a comparable increase in VDR level by TGFß in several osteoblast cell lines and in isolated fetal rat osteoblasts, which indicates that this TGFß effect is not specific for transformed cells and is independent of species and state of differentiation. Data obtained with ROS 17/2.8 cells indicate that the effect of TGFß is independent of basal VDR level. The increase in apparent VDR level can be the

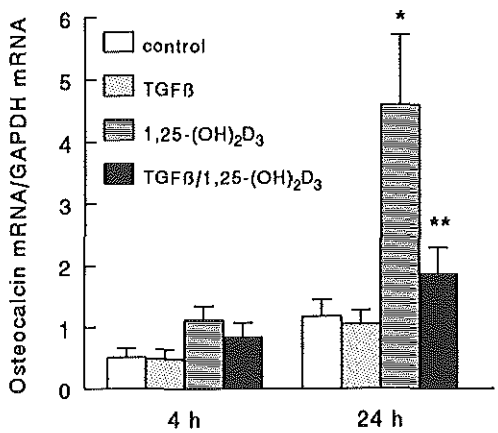

Figure 7

Effect of preincubation with TGFß on 4 and 24 h 1,25-$(OH)_2D_3$-increased osteocalcin mRNA in ROS 17/2.8 cells. Cells were cultured as described in Materials and Methods and incubated for 4 h with 1 ng/ml TGFß and subsequently (without medium change) for 4 h and 24 h with 10^{-9}M 1,25-$(OH)_2D_3$. Northern blots were quantitated by densitometric scanning. Data are expressed as the ratio of the amount of osteocalcin mRNA to that of GAPDH mRNA. * $p<0.05$ versus control. ** $p<0.05$ calculated as significance of interaction.

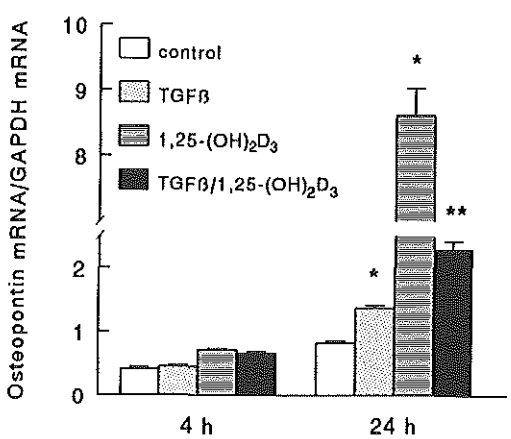

Figure 8

The effect of preincubation with TGFß on 1,25-$(OH)_2D_3$-induced increase in osteopontin mRNA in ROS 17/2.8 cells. Cells were cultured as described in Materials and Methods and incubated for 4 h with 1 ng/ml TGFß and subsequently (without medium change) for 4 h and 24 h with 10^{-9} M 1,25-$(OH)_2D_3$. Northern blots were quantitated by densitometric scanning. Data are expressed as the ratio of the amount of osteopontin mRNA to that of GAPDH mRNA. * $p<0.001$ versus control. ** $p<0.001$ calculated as significance of interaction.

result of 1) an increase in receptor affinity, 2) an increase in new receptor synthesis, and/or 3) increase in half-life of the receptor or its mRNA. Scatchard analysis revealed that TGFß increases the VDR number without a change in the apparent dissociation constant. The effect of actinomycin D on TGFß-stimulated 1,25-$(OH)_2D_3$ binding indicates an effect on new synthesis of VDR rather than on VDR half-life and this is confirmed by mRNA analysis. Although we did not perform nuclear run-on experiments the fact that actinomycin D completely abolishes the TGFß-induced increase in VDR mRNA level suggests that the TGFß effect is mainly the result of stimulation of VDR gene transcription and not of an increased VDR mRNA half-life. However, additional posttranscriptional effects can not be excluded.

In both NIH 3T3 fibroblasts and ROS 17/2 cells TGFß activates the α2(1) collagen promoter via a specific sequence in the promoter that is the binding site for nuclear factor I (NF1) (36). In view of this the current data are suggestive for the presence of a TGFß-responsive element or NF1 binding site in the VDR gene. However, as the sequence of the 5'-flanking region of the rat VDR gene is yet unknown it was not possible to identify such sequences in the VDR gene and also within the known 115 nucleotides of the promoter region of the human VDR gene (37) no NF1 binding site could be detected. Kim et. al (38) demonstrated that TGFß and phorbol ester act through the same site in the promoter of the TGFß-gene. It is not likely that TGFß and phorbol ester/protein kinase C act through the same site in the VDR gene as 4 h phorbol ester treatment results in a decrease (39), whereas TGFß causes an increase in VDR mRNA.

For several 1,25-$(OH)_2D_3$ responses a relation with VDR level has been described (8,12-14). The present study shows that although TGFß increases VDR level it potently inhibits 1,25-$(OH)_2D_3$-stimulated osteocalcin production (Figs. 6A and B). This dissociation is not unique for osteocalcin since the 1,25-$(OH)_2D_3$-induced increase in osteopontin mRNA was also inhibited by TGFß (Fig. 8). Since we initially studied the effect of TGFß preincubation on 24 h stimulation by 1,25-$(OH)_2D_3$, it was conceivable that during 24 h of incubation postreceptor mechanisms induced by TGFß were more important than VDR up-regulation. These postreceptor mechanisms might be absent or less important in rapid responses to 1,25-$(OH)_2D_3$. However, time course studies revealed that an inhibitory effect of TGFß could be observed as soon as an effect of 1,25-$(OH)_2D_3$ on osteocalcin production was detected. Although not yet significant, the inhibitory effect of TGFß pretreatment on 1,25-$(OH)_2D_3$-induced increase in osteocalcin mRNA was already observed after 4 h incubation with 1,25-$(OH)_2D_3$. These data obtained with the TGFß preincubation studies show an interaction between TGFß and 1,25-$(OH)_2D_3$ with respect to osteocalcin and osteopontin mRNA synthesis at another level than VDR regulation.

This is supported by the observations that 0.1 ng/ml TGFß caused a significant increase in VDR level, whereas it did not affect 1,25-$(OH)_2D_3$-induced osteocalcin production.

Our results show interaction between TGFß and 1,25-$(OH)_2D_3$ in the regulation of osteocalcin production. Osteocalcin is a chemoattractant for human peripheral blood monocytes and macrophages (40,41) and data have been reported that osteocalcin is involved in recruitment and/or differentiation of bone resorbing cells (42,43). Recently, it has been shown that TGFß, at doses effective in the present study, inhibits 1,25-$(OH)_2D_3$-induced formation of osteoclast-like cells in human and mouse bone marrow cultures (44,45). Combining these data from literature and the current data on the potent inhibitory effect of TGFß on 1,25-$(OH)_2D_3$-stimulated osteocalcin production it is tempting to postulate that TGFß inhibits 1,25-$(OH)_2D_3$-induced osteoclast formation via inhibition of 1,25-$(OH)_2D_3$-stimulated osteocalcin synthesis. Since osteopontin has been suggested to act as an anchor of osteoclasts to bone (46,47), our data on osteopontin support the idea of a negative interaction between TGFß and 1,25-$(OH)_2D_3$ in the regulation of osteoclast action and are thereby in line with the osteocalcin data.

In conclusion the present study demonstrates a TGFß-induced dissociation between VDR level and 1,25-$(OH)_2D_3$ action in osteoblast-like cells. At the moment the significance of VDR up-regulation by TGFß remains unclear. Whether this is important for other 1,25-$(OH)_2D_3$ biological responses has to be established. More generally, the present study demonstrates the importance of the presence of locally produced growth factors for the eventual response of target tissues/cells to hormone treatment.

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CHAPTER 3

ANTAGONISTIC EFFECTS OF TGFβ ON VITAMIN D_3 ENHANCEMENT OF OSTEOCALCIN AND OSTEOPONTIN TRANSCRIPTION: REDUCED INTERACTIONS OF VITAMIN D RECEPTOR/RETINOIC X RECEPTOR COMPLEXES WITH VITAMIN D RESPONSE ELEMENTS

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Endocrinology, in press

ABSTRACT

Osteocalcin and osteopontin are non-collagenous proteins secreted by osteoblasts and regulated by a complex interplay of systemic and locally produced factors, including growth factors and steroid hormones. We investigated the mechanism by which transforming growth factor ß (TGFß) inhibits 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) enhanced expression of the osteocalcin (OC) and osteopontin (OP) genes. ROS 17/2.8 cells, in which both genes are expressed, were transfected with reporter constructs driven by native (*i.e.* wild-type) rat OC and mouse OP promoters. TGFβ abrogated the $1,25(OH)_2D_3$ enhanced transcription of both the OC and OP genes. The inhibitory TGFβ response for each requires Vitamin D response element (VDRE) sequences, although there are additional contributions from proximal basal regulatory elements. These transcriptional effects were further investigated for contribution of the *trans*-activating factors, which interact with OC and OP VDREs, involving the vitamin D receptor (VDR) and retinoic X receptor (RXR). Gel mobility shift assays show that TGFß significantly reduces induction of the heterodimeric VDR/RXR complexes in $1,25(OH)_2D_3$ treated ROS 17/2.8 cells. However, Western blot and ligand binding analyses reveal that TGFß does not affect nuclear availability of the VDR. We also show that activator protein-1 activity is up-regulated by TGFβ; thus, activator protein-1 binding sites in the OC promoter may potentially contribute to inhibitory effects of TGFβ on basal transcription. Our studies demonstrate that the inhibitory action of TGFβ on the $1,25(OH)_2D_3$ enhancement of OC and OP transcription in osteoblastic cells results from modulations of protein-DNA interactions at the OC and OP VDRE, which can not be accounted for by changes in VDR protein levels. As OC and OP participate in bone turnover, our results provide insight into the contributions of TGFβ and $1,25(OH)_2D_3$ to VDR mediated gene regulatory mechanisms operative in bone formation and/or resorption events.

INTRODUCTION

Osteocalcin (OC) and osteopontin (OP) are two major non-collagenous calcium binding proteins in bone (1). During development of the osteoblast phenotype osteopontin is expressed during the period of active proliferation, decreases postproliferatively and expression is upregulated in mature osteoblasts. In contrast, osteocalcin is expressed only postproliferatively in mature osteoblasts with the onset of

extracellular matrix mineralization (2). In ROS 17/2.8 osteosarcoma cells, however, both osteocalcin and osteopontin are constitutively expressed. Some evidence points to the involvement of both proteins in bone turnover; for example, both proteins bind to hydroxyapatite and are regulated by $1,25(OH)_2D_3$ and TGFβ. In addition, osteocalcin contributes to recruitment and/or differentiation of osteoclasts (3-5) and osteopontin appears to be important for attachment of the osteoclasts to the bone matrix (6, 7).

TGFβ and $1,25(OH)_2D_3$ are involved in regulation of both bone formation and resorption. The steroid hormone $1,25(OH)_2D_3$ plays a principal role in calcium homeostasis and skeletal metabolism (8), as well as influences expression of genes related to establishment and maintenance of the bone cell phenotype (9). $1,25(OH)_2D_3$ exerts its primary effect by binding to its nuclear receptor, the vitamin D receptor (VDR), which belongs to the steroid/retinoid/thyroid hormone receptor superfamily (10) and acts via binding to distinct vitamin D response elements (VDREs). VDREs have been identified in several genes related to osteoblastic differentiation and function: human osteocalcin (11, 12), rat osteocalcin (13-15), mouse osteopontin (16), rat 25-hydroxy- and 1,25-dihydroxyvitamin D_3 24-hydroxylase (17-20), calbindin D28k (21), and $ß_3$ integrin (22).

TGFß is locally produced in bone by osteoblasts and osteoclasts and represents a member of the TGFß/BMP superfamily of polypeptide hormones, which play key roles in skeletal development and bone remodeling (23, 24). In mammals three isoforms of TGFβ have been identified: $TGFß_1$, $TGFß_2$ and $TGFß_3$ (25, 26). TGFß is present as a latent complex in bone and released from the extracellular matrix in an active form during bone resorption by osteoclasts (27). TGFß modulates the expression of several markers of the osteoblast phenotype (28-35).

Recent studies suggest that regulation of bone metabolism by $1,25(OH)_2D_3$ and TGFß is functionally coupled. For example, combined treatment with TGFß and $1,25(OH)_2D_3$ of both human and rat osteoblast-like cells has been shown to affect parameters of osteoblastic differentiation and function (36-38). However, the mechanisms by which TGFß and $1,25(OH)_2D_3$ together modulate the expression of osteoblast-related genes remains to be established. In this study we examined the antagonistic effects of $TGFß_1$ and $TGFß_2$ on the $1,25(OH)_2D_3$ enhancement of osteocalcin and osteopontin gene transcription. We show that TGFβ inhibition of $1,25(OH)_2D_3$ stimulated transcription involves the selective down-regulation of VDR/RXR interactions with both the OC and OP VDREs.

MATERIALS AND METHODS

Cell culture

ROS 17/2.8 osteosarcoma cells, kindly provided by Dr. G. Rodan and Dr. S. Rodan (Merck Sharp & Dohme Research Laboratories, West Point, PA) were maintained in Ham's F12 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 5% FCS at 37 °C under 95% air/5% CO_2. For the experiments, cells were seeded in 100 x 20 mm plastic dishes at 0.7 x 10^6 cells/plate in Ham's F12 medium containing 5% FCS and cultured for 24 h. Subsequently, medium was replaced by Ham's F12 with 2% charcoal-treated FCS and cells were cultured overnight and then incubated for 24 h with 10^{-8} M $1,25(OH)_2D_3$ and/or 2.5 ng/ml of either $TGFß_1$ or $TGFß_2$. $1,25(OH)_2D_3$ was kindly provided by Dr. M. Uskokovic, Hoffman-La Roche Inc. (Nutley, NJ). $TGFß_1$ was purchased from R&D Systems (Minneapolis, MN). $TGFß_2$ was kindly provided by Dr. J. Feyen, Sandoz Pharma LtD (Basel, Switzerland). Medium was analyzed for osteocalcin by RIA as previously described (39).

Gel mobility shift assay

Nuclear extracts were prepared as previously described (40). In brief, cells were scraped in PBS and after centrifugation the cell pellet was reconstituted in a hypotonic lysis buffer (10 mM HEPES, pH 7.5, 10 mM KCl and 0.2 mM EDTA) supplemented with 0.75 mM spermidine, 0.15 mM spermine, 1 mM EGTA, 1 mM dithiothreitol and a broad spectrum of protease inhibitors: 0.2 mM PMSF, 70 μg/ml TPCK, 10 μg/ml trypsin inhibitor, 0.5 μg/ml leupeptin and 1.0 μg/ml pepstatin. Then 0.5% Nonidet P40 was added and cells were lysed using a dounce homogenizer. After centrifugation, nuclear extracts were obtained by incubating nuclei in a hypertonic nuclear extraction buffer (20mM HEPES, pH 7.5, 420 mM KCl, 25% glycerol and 0.2 mM EDTA) supplemented with the same reagents previously mentioned for the hypotonic lysis buffer. Desalting was performed by dilution with hypertonic nuclear extraction buffer without KCl.
For gel mobility shift assays the following oligonucleotides were synthesized:
rat osteocalcin VDRE (14): 5'-CTGCACTGGGTGAATGAGGACATTACTGA-3';
mouse osteopontin VDRE (16): 5'-ACAAGGTTCACGAGGTTCACGTCT-3';
metallothionein II/activator protein-1 (AP-1) consensus: 5'-AATTCGTGACTCAGCGCGCG-3';
SP-1 consensus: 5'-CGGATGGGCGGGGCCGGGGATGGGCGGGGCCGG-3'.

Gel-purified oligonucleotides were radiolabeled with [γ^{32}P] ATP as described previously (41). Protein/DNA binding reactions were performed by combining 10 μl of a 5 μg protein mixture in a final KCl concentration of 100 mM with 10 μl of a DNA mixture containing 1 fmol/μl DNA, 0.2 μg/μl nonspecific competitor DNA (poly(dI-dC)*(dI-dC)) and 2 mM dithiothreitol for 15 min. Protein/DNA complexes were separated in a 4% polyacrylamide gel (acrylamide:bisacrylamide=80:1) in 0.5x TBE electrophoresis buffer. Gels were dried and exposed to x-ray film.

Transient transfection assay

The OC chloramphenicol acetyltransferase (CAT) chimeric reporter gene constructs pOCZCAT and *Bgl*IICAT contain the osteocalcin promoter sequences -1097/+23 and -348/+23, respectively (42). The OP CAT chimeric reporter gene constructs pOP772CAT and pOP552CAT span nucleotides -772/+40 and -552/+41, respectively (16). The $(VDRE)_3$-tk-luciferase chimeric reporter construct, which was generously

provided by Dr. L.J. Sturzenbecker (Hoffman-La Roche), contains three copies of the rat osteocalcin VDRE fused to thymidine kinase luciferase. DEAE-dextran mediated transfections (43) were performed with cells plated at a density of 0.8 x 10^5 per well in 6-well plates. CAT and luciferase reporter gene constructs (5 μg) were either cotransfected with 2 μg Rous sarcoma virus LTR luciferase or pSV2CAT (used as an internal control for transfection efficiency) 48 h after plating. After transfection, cells were fed with Ham's F12 medium supplemented with 5% FCS. Subsequently, 4 h after feeding, cells were incubated with $TGFß_1$, $TGFß_2$ and/or $1,25(OH)_2D_3$ in Ham's F12 with 2% charcoal-treated FCS. Cells were harvested after incubation for 24 h and CAT activity was determined (43). CAT and luciferase assays were normalized on the basis of luciferase activity and CAT activity, respectively, as well as protein content by Bradford assay (Pierce). Quantitation was performed by using a ß-emission analyzer (Betascope 603 Betagen).

Northern blot analysis

RNA isolation, electrophoresis of total RNA (10 μg) in formaldehyde gels, Northern blotting [Zetaprobe membranes (Biorad)], prehybridizations and hybridizations were performed as described previously (44). The sources of the following probes used for hybridization are described by Shalhoub *et al.* (44): osteocalcin, osteopontin, histone H4 , collagen type I, alkaline phosphatase, and vitamin D receptor probes. Values for mRNA were normalized to GAPDH and ribosomal 28S transcripts. For rehybridization, filters were washed for 2 h at 65 °C with 5 mM Tris (pH 8.0), 0.2 mM EDTA (pH 8.0), 0.05% sodiumpyrophosphate and 0.1 x Denhardt's solution.

Western blot analysis

Nuclear proteins were separated in 12% SDS-PAGE gels (acrylamide:bisacrylamide=30:1) (43) and transferred to Zetaprobe membrane followed by Western blot analysis (ECL Amersham). In brief, 5% blocking agent was used in PBS containing 0.1% Tween-20. The blot was subsequently incubated with VDR antibody (IVG8C11) in a 1:1000 dilution and incubated with the second antibody (peroxidase anti-mouse antibody at a 1:10,000 dilution).

Preparations of cell extracts and $[^3H]1,25(OH)_2D_3$ binding assay

Vitamin D ligand binding assays were performed as described by Pols *et al.* (45). In brief, after incubation with TGFß and/or $1,25\text{-}(OH)_2D_3$, cells were washed with 2% BSA in serum-free medium for 2 h to remove residual unlabeled $1,25\text{-}(OH)_2D_3$. Then cells were extracted on ice in a hypertonic buffer consisting of 300 mM KCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM dithiothreitol, 10 mM sodium molybdate and 0.1% Triton X-100. High-speed supernatants were obtained, and 250 μl aliquots were incubated overnight at 4 °C for single point assays with 0.25 nM $[^3H]1,25(OH)_2D_3$ in the absence or presence of a 200-fold molar excess of unlabeled hormone. After removal of unbound ligand, receptor-bound vitamin D was quantitated by liquid scintillation counting.

Data analysis

To assess significance of the interaction between two agents, tested data were analyzed with analysis of variance for two-way design (indicated by #). Other statistical analyses were performed using Student's *t* test (indicated by *)

RESULTS

Effects of TGFβ$_1$, TGFβ$_2$ and 1,25(OH)$_2$D$_3$ on expression of genes related to osteoblast function and differentiation.

As osteoblasts are responsive to TGFß$_1$ and TGFß$_2$, and display three classes of TGFß receptors (46), we explored possible differential effects of these two major isoforms present in bone. Northern blot analysis reveals inhibitory effects of both TGFβ$_1$ and TGFβ$_2$ on basal osteocalcin gene expression (Fig. 1). Steady state mRNA levels of osteopontin were not affected by either growth factor after 24 h of treatment. The 24-h time point was selected for our studies as this treatment interval facilitates assessment of the combined effects of TGFß and 1,25-(OH)$_2$D$_3$ on OC and OP gene expression. We have previously established that under our experimental conditions, 1,25-(OH)$_2$D$_3$ enhancement of OC gene expression is maximal at 24 h after adminstration of the ligand (9). Also, although TGFß modulates parameters of 1,25-(OH)$_2$D$_3$-mediated gene expression as a component of an earlier response (*i.e.* 6 h), maximal effects of TGFß on OC gene expression are observed at the 24-h time point (37).

The combined effects of TGFß and 1,25-(OH)$_2$D$_3$ on OC and OP gene expression are also shown in Fig. 1. Both OC and OP mRNA levels were increased 6- to 10-fold after 24 h of 1,25-(OH)$_2$D$_3$ treatment. Both TGFß$_1$ and TGFß$_2$ inhibited the 1,25-(OH)$_2$D$_3$-induced increases in OC or OP expression. However, the magnitude of the decrease was reproducibly greater for osteocalcin in multiple experiments, consistent with the influence of TGFß on basal level of OC gene expression. For 1,25-(OH)$_2$D$_3$-treated cells, the level of secreted OC in the medium, which reflects OC protein synthesis, parallels the changes in OC gene expression (Table 1). For comparison, TGFβ and 1,25-(OH)$_2$D$_3$ effects on other bone-related proteins (*e.g.* alkaline phosphatase, collagen type 1 and vitamin D receptor) and cell proliferation marker (histone H4) were determined. These genes display different patterns of expression after TGFß and 1,25(OH)$_2$D$_3$ treatment, demonstrating the selective responsiveness of the OC and OP genes to these factors.

Effects of combined treatment with TGFβ and 1,25(OH)$_2$D$_3$ on OC and OP transcription.

1,25-(OH)$_2$D$_3$ regulates OC and OP gene expression by specific interactions of VDR containing transcription factor complexes with defined VDREs in the promoters of both genes. To gain insight into the mechanism by which TGFß inhibits 1,25-(OH)$_2$D$_3$ enhancement of OC and OP gene expression, we performed transient

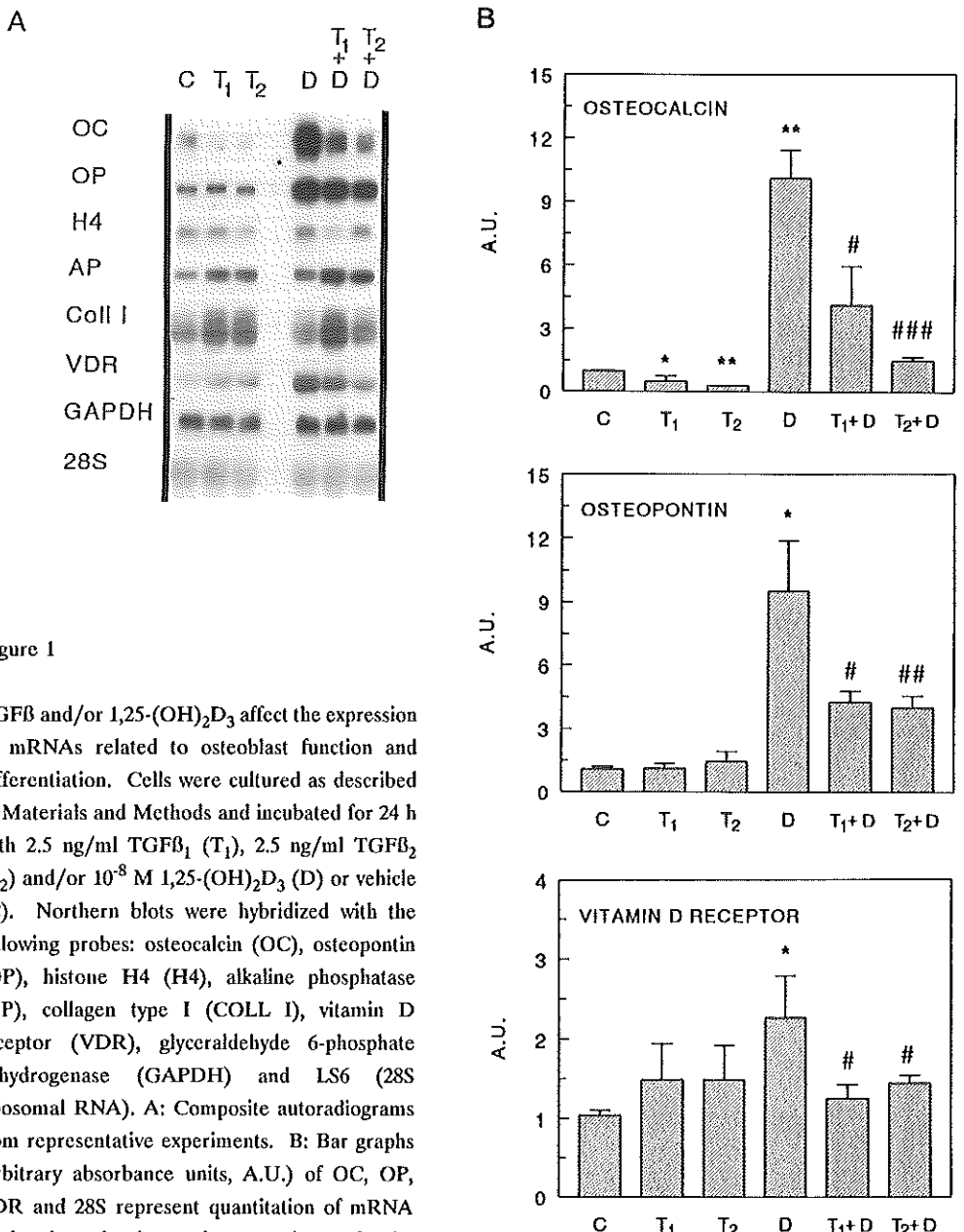

Figure 1

TGFß and/or 1,25-$(OH)_2D_3$ affect the expression of mRNAs related to osteoblast function and differentiation. Cells were cultured as described in Materials and Methods and incubated for 24 h with 2.5 ng/ml $TGFß_1$ (T_1), 2.5 ng/ml $TGFß_2$ (T_2) and/or 10^{-8} M 1,25-$(OH)_2D_3$ (D) or vehicle (C). Northern blots were hybridized with the following probes: osteocalcin (OC), osteopontin (OP), histone H4 (H4), alkaline phosphatase (AP), collagen type I (COLL I), vitamin D receptor (VDR), glyceraldehyde 6-phosphate dehydrogenase (GAPDH) and LS6 (28S ribosomal RNA). A: Composite autoradiograms from representative experiments. B: Bar graphs (arbitrary absorbance units, A.U.) of OC, OP, VDR and 28S represent quantitation of mRNA levels by densitometric scanning of the autoradiogram. OC, OP and VDR mRNA levels are normalized for 28S. Similar results were obtained in multiple independent experiments.

* P<0.05, ** P<0.001 versus control. # P<0.05, ## P<0.01, ### P<0.005 calculated as significance of interaction.

TABLE 1

Effect of either $TGF\beta_1$ or $TGF\beta_2$ and $1,25\text{-}(OH)_2D_3$ on osteocalcin secretion in medium of ROS 17/2.8 cells (ng/ml).

	control	$TGF\beta_1$	$TGF\beta_2$
control	2.76 ± 0.23	2.13 ± 0.44	2.26 ± 0.40
$1,25(OH)_2D_3$	15.45 ± 2.15	3.44 ± 0.35	3.14 ± 0.08

Cells were cultured as described (Materials and methods) and incubated for 24 h with 2.5 ng/ml $TGF\beta_1$, 2.5 ng/ml $TGF\beta_2$ and/or 10^{-8} M $1,25\text{-}(OH)_2D_3$. Data are expressed as mean ± SD.

transfections assays in ROS 17/2.8 cells with OC and OP promoter CAT fusion constructs in the presence of TGFß and/or $1,25\text{-}(OH)_2D_3$. After transfection with an OC promoter construct of 1100 nucleotides, which contains the VDRE (pOCZCAT; Fig. 2A), we observed a 4- to 6-fold decrease in basal CAT activity in response to both $TGF\beta_1$ and $TGF\beta_2$. $1,25\text{-}(OH)_2D_3$ increased basal CAT activity 8-fold in 24 h. This increase was inhibited 20- to 30-fold after coincubation with either $TGF\beta_1$ or $TGF\beta_2$. Thus, in addition to a significant inhibition of basal transcription, both TGFß subtypes abrogated $1,25(OH)_2D_3$ enhanced OC transcription.

To ascertain the extent to which TGFβ effects on OC depend on VDRE sequences, ROS 17/2.8 cells were transfected with a construct in which the VDRE was deleted (p*Bgl*IICAT; Fig. 2B). As expected, the construct did not respond to $1,25\text{-}(OH)_2D_3$. $TGF\beta_1$ or $TGF\beta_2$ either alone or in the presence of $1,25\text{-}(OH)_2D_3$ caused a 4- to 6-fold decrease in CAT activity. This inhibitory effect of TGFβ on basal OC gene transcription is consistent with the presence of a TGFβ response element in the proximal promoter (47). Notably, the fold decrease resulting from coincubation of TGFß and $1,25\text{-}(OH)_2D_3$ was significantly less for the OC promoter CAT construct lacking the VDRE (4- to 6-fold; p*Bgl*IICAT) as compared to the OC CAT construct containing the VDRE (20- to 30-fold; pOCZCAT). To assess the promoter selectivity of TGFß effects on transcription of bone-related genes, we performed similar experiments with CAT reporter gene constructs fused to the SV40 promoter (pSV2CAT) or lacking a promoter (pSV0CAT). TGFß has only modest inhibitory effects on the virally derived SV40 promoter (1.3-fold; data not shown).

Similar to results with the OC promoter, transfection with the osteopontin promoter construct pOP772CAT (Fig. 3A), containing 772 nt of 5' flanking

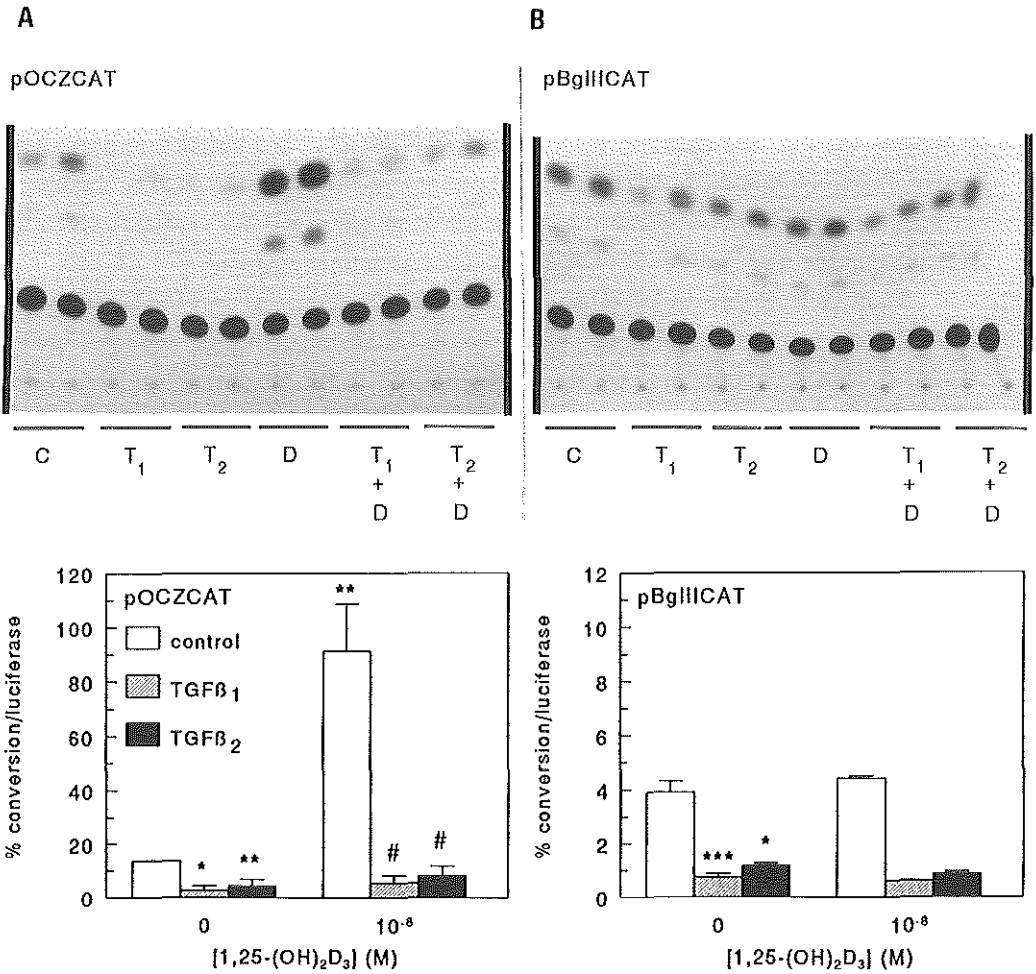

Figure 2

$TGF\beta_1$ and $TGF\beta_2$ antagonize basal and $1,25(OH)_2D_3$ stimulated OC transcription. ROS 17/2.8 cells were transiently transfected with the following osteocalcin promoter-CAT fusion constructs: A) pOCZCAT, -1097/+23, and B) p*Bgl*IICAT -348/+23. Cells were transfected and 4 h later incubated for 24 h in the absence (control) or presence of 2.5 ng/ml TGFß1 (T_1), or TGFß2 (T_2). These treatment groups were coincubated with either vehicle (C, 0) or $1,25\text{-}(OH)_2D_3$ (D, 10^{-8} M). Upper portions of each panel show one representative autoradiogram of a CAT assay. The lower portion of each panel are bar graphs representing mean ± SD of duplicates and triplicates from three independent transfection experiments after quantitation using a ß-emission analyzer. CAT activity was corrected for transfection efficiency by luciferase activity. * $P<0.001$, ** $P<0.005$, *** $P<0.01$ versus control. # $P<0.01$ calculated as significance of interaction.

sequences, showed a decrease in basal CAT activity after incubation with either TGFß$_1$ or TGFß$_2$ (2-fold). 1,25-$(OH)_2D_3$ caused an 11-fold increase in CAT activity, which was inhibited 7-fold by TGFß$_1$ and TGFß$_2$. The OP construct lacking the VDRE (pOP552CAT; Fig. 3B) does not respond to 1,25-$(OH)_2D_3$. We noted that a modest level of TGFβ inhibition (2-fold) of OP basal promoter activity persists. Taken together, our results suggest that TGFβ exerts a major effect on 1,25-$(OH)_2D_3$ mediated OC and OP transcription and requires a VDRE containing promoter segment.

To investigate directly the effect of TGFß and 1,25-$(OH)_2D_3$ on VDRE-mediated transcription, we transfected cells with the $(VDRE)_3$-tk-luciferase reporter gene construct, which contains three copies of the rat OC VDRE (Fig. 4). Treatment with 1,25-$(OH)_2D_3$ increased basal luciferase activity approximately 4-fold in 24 h. This increase was inhibited 50% and 30% by TGFß$_1$ and TGFß$_2$, respectively, indicating that the inhibitory effect of TGFß on 1,25-$(OH)_2D_3$ enhancement of OC transcription is at least partly mediated by the VDRE.

TGFβ and 1,25-$(OH)_2D_3$ modulate protein/DNA interactions at VDREs

To address directly the mechanism by which TGFß abrogates 1,25-$(OH)_2D_3$-dependent enhancement of OC transcription, we examined protein/DNA interactions at VDREs. Gel shift assays were performed with nuclear proteins from ROS 17/2.8 cells treated with TGFß and/or 1,25-$(OH)_2D_3$, using labeled OC and OP VDRE oligonucleotides. Fig. 5 shows the well-established induction of the VDR containing complexes at both OC and OP VDREs after treatment of ROS 17/2.8 cells with 1,25-$(OH)_2D_3$ (16, 40, 48-50). Nuclear proteins from control or TGFβ_2-treated cells do not show VDRE protein/DNA interactions. However, combined treatment of 1,25-$(OH)_2D_3$ with TGFß caused a significant reduction of the VDR containing complex at both the OC and OP VDREs. Similar reductions in VDRE complexes were observed in TGFβ_1 treated cells (data not shown). This indicates that the inhibitory effects by TGFß$_1$ and TGFβ_2 on the 1,25-$(OH)_2D_3$-induced increase in OC and OP transcription are at least in part due to decreased binding of transcription factors to the VDRE.

Because both TGFβ and 1,25-$(OH)_2D_3$ modulate expression of the fos and jun family of proto-oncogenes in osteoblasts (34, 51-53), we also examined the effects of these agents on AP-1 activity in ROS 17/2.8 osteosarcoma cells. Gel shift assays were performed using AP-1 and SP-1 consensus sequences as the probes (Fig. 6).
The AP-1 complex was increased after incubation with either TGFß or 1,25-$(OH)_2D_3$, although the increase observed with TGFß was more pronounced than with

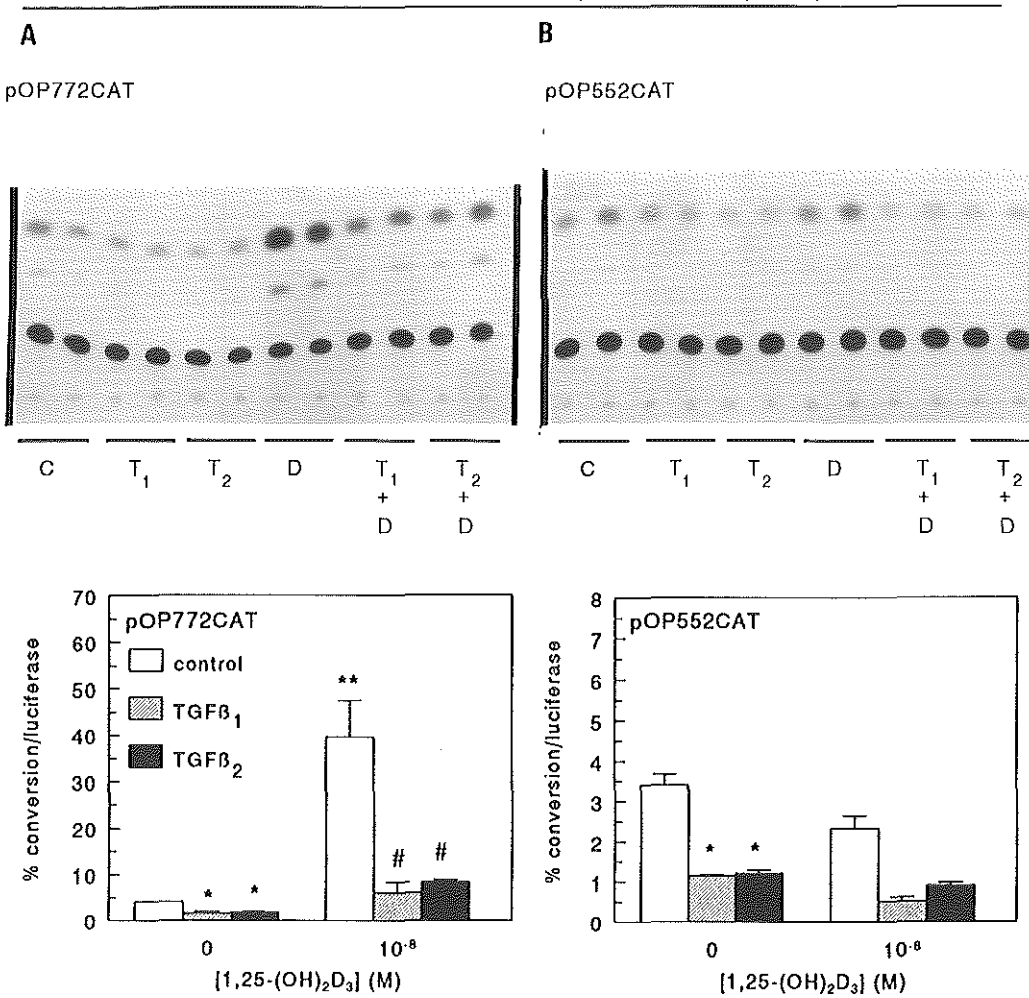

Figure 3

$TGF\beta_1$ and $TGF\beta_2$ antagonize basal and $1,25(OH)_2D_3$ stimulated OP transcription. ROS 17/2.8 cells were transiently transfected with the following osteopontin promoter-CAT fusion constructs: panel A) pOP772CAT, -772/+40, panel B) pOP552CAT -552/+40. Cells were transfected and 4 h later incubated for 24 h in the absence (control) or presence of 2.5 ng/ml TGFß1 (T_1), or TGFß2 (T_2). These treatment groups were co-incubated with either vehicle (C, 0) or $1,25-(OH)_2D_3$ (D, 10^{-8} M). Upper portion of each panel shows one representative autoradiogram of a CAT assay. The lower portion of each panel are bar graphs representing mean ± SD of duplicates and triplicates from three independent transfection experiments after quantitation using a ß-emission analyzer. CAT activity was corrected for transfection efficiency by luciferase activity. * $P<0.001$, ** $P<0.005$ versus control. # $P<0.01$ calculated as significance of interaction.

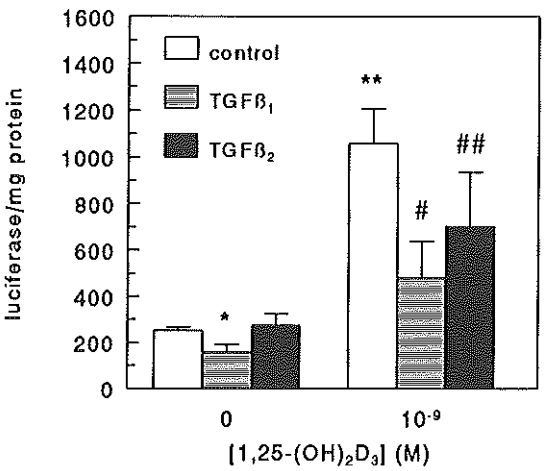

Figure 4

$TGFß_1$ and $TGFß_2$ inhibit $1,25\text{-}(OH)_2D_3$ enhancement of OC VDRE-mediated transcription. ROS 17/2.8 cells were transiently transfected with the $(VDRE)_3$-tk-luciferase construct. Cells were transfected and 4 h later incubated for 24 h in the absence or presence of 2.5 ng/ml $TGFß_1$ and $TGFß_2$. These treatment groups were coincubated with either vehicle or $1,25\text{-}(OH)_2D_3$ (10^{-8} M). Bar graphs represent mean ± SD of luciferase activity. * $P<0.05$, ** $P<0.005$ versus control. # $P<0.01$, ## $P<0.05$ calculated as significance of interaction.

$1,25(OH)_2D_3$. Combined treatment caused a further enhancement of the AP-1 complex. No modulation of SP-1 binding activity was observed, which represents an internal control for protein quantitation. These findings indicate selectivity of the TGFβ- and $1,25\text{-}(OH)_2D_3$-mediated signaling mechanisms that modulate nuclear transcription factor levels.

VDR protein levels are not rate-limiting for VDRE interactions in the presence of TGFβ and $1,25\text{-}(OH)_2D_3$

Because changes in VDR mRNA levels were observed after treatment with $TGFß_1$, $TGFß_2$ and $1,25\text{-}(OH)_2D_3$ (Fig. 1), we studied the effects of $TGFß_1$ and $TGFß_2$ in the absence or presence of $1,25\text{-}(OH)_2D_3$ on VDR protein levels, as a possible mechanism for the inhibitory effects of TGFß on VDR/RXR interactions at the OC and OP VDREs. We investigated the effect of TGFß on homologous up-regulation of the VDR by western blot analysis. The results indicate that nuclear VDR levels are only detected after treatment with $1,25\text{-}(OH)_2D_3$, and that neither $TGFβ_1$ nor $TGFß_2$ affects

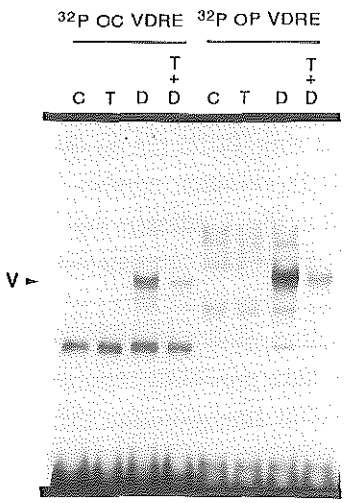

Figure 5

TGFß modulates interactions of endogenous 1,25-$(OH)_2D_3$ inducible VDR/RXR complexes from ROS 17/2.8 cells. Binding of nuclear proteins to the OC VDRE and OP VDRE was analyzed in gel mobility shift assays. Cells were incubated for 24 h with vehicle (C), 2.5 ng/ml $TGFß_2$ (T), 10^{-8} M 1,25-$(OH)_2D_3$ (D) or both (T+D). Nuclear protein preparations were incubated with either radiolabeled OC- or OP-VDRE. The vitamin D responsive complexes (V) for the OC- and OP-VDRE are indicated by the arrowhead.

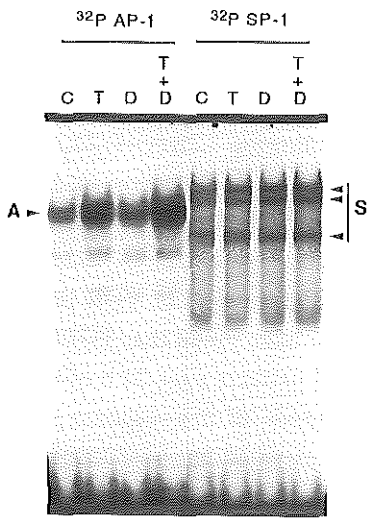

Figure 6

Selective enhancement of AP-1 activity by TGFß and 1,25-$(OH)_2D_3$. Gel mobility shift assays were performed with AP-1 and SP-1 consensus oligonucleotides. Nuclear extracts were identical to those in Fig. 5. Abbreviations and symbols are as indicated in Fig. 5. The AP-1 complexes (A) represent a composite of heterodimers of the fos and jun family members and are indicated by the arrowhead. The triple arrowhead (S) indicates three specific complexes mediated by the SP-1 family of transcription factors; the upper complex represents SP-1 and the two lower complexes are SP-3 related proteins.

the 1,25-$(OH)_2D_3$ dependent increase in VDR levels (Fig. 7A). Subtle variations in the 1,25-$(OH)_2D_3$-treated lanes appear to reflect similar variations in the nonspecific band migrating slower than the VDR. The lack of an effect by TGFß on VDR protein levels in the presence of 1,25-$(OH)_2D_3$ is further corroborated by quantitative analysis of VDR protein using immunoradiometric assays; homologous up-regulation of VDR number in

nuclear extracts after 1,25-$(OH)_2D_3$ treatment was not affected by cotreatment with either $TGF\beta_1$ or $TGF\beta_2$ (data not shown). Ligand binding assays in whole cell extracts using isotopically labeled 1,25-$(OH)_2D_3$ (Fig. 7B) also demonstrate homologous up-regulation of 1,25-$(OH)_2D_3$-receptor number, as reflected by a 5-fold increase in ligand binding following 1,25-$(OH)_2D_3$ treatment. Cotreatment with TGFß (24 h) does not affect the the homologous up-regulation of 1,25-$(OH)_2D_3$-receptor number in the presence of 1,25-$(OH)_2D_3$. Thus, the evidence presented in Fig. 7 indicates that TGFβ inhibition of protein/DNA interactions at the VDRE is not the consequence of a reduction in the level of VDR protein.

We have found that TGFß alone causes a subtle enhancement of VDR levels in whole cell extracts after 24 h of treatment ($P < 0.05$). We have previously reported that the magnitude of this response is higher at 6 h after TGFß administration (37). The modestly elevated levels of VDR protein after 24 h of TGFß treatment therefore may represent a residual, and perhaps persistent, component of TGFß responsiveness. Our previous observation that TGFß enhances VDR levels in whole cell extracts as a component of an earlier response (*i.e.* 6 h)(37) and our current finding that show that TGFß does not significantly influence homologously up-regulated levels of the VDR protein at 24 h are both consistent with a complex temporal regulatory mechanism for modulation of the VDR levels that may in part be compartmentalized.

DISCUSSION

In this study we focused on gene regulatory mechanisms that control OC and OP expression which are affected by the combined activities of 1,25-$(OH)_2D_3$ and TGFß. The basis of our studies is supported by previous studies (36, 37), and our present findings which show that TGFß inhibits the 1,25-$(OH)_2D_3$-induced increase of both OC and OP mRNA levels. The physiological relevance of TGFβ inhibition of the 1,25-$(OH)_2D_3$ enhanced expression of osteoblast-related genes is exemplified by decreased secretion of the osteocalcin protein, a major functional product of osteoblasts. In addition, by directly comparing the actions of $TGF\beta_1$ and $TGF\beta_2$, we show that these agents exert identical effects on regulation of OC and OP gene expression. This finding is consistent with the observation that $TGF\beta_1$- and $TGF\beta_2$-mediated cell signaling pathways operate via shared TGFβ receptor classes (46).

Using transfection assays with osseous cells and reporter gene constructs containing the native OC and OP gene promoters, we establish that 1,25-$(OH)_2D_3$ related

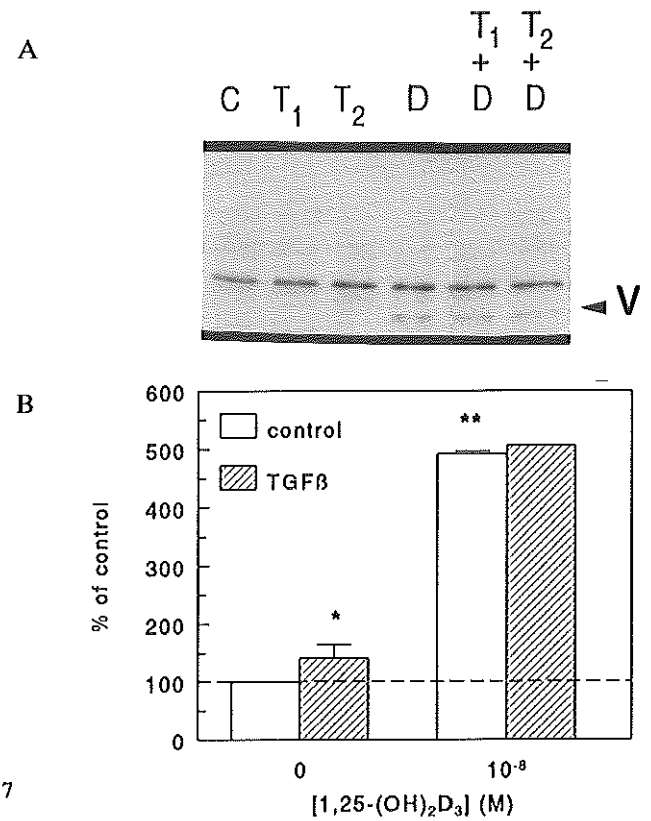

Figure 7

Homologous upregulation of VDR protein levels by 1,25-$(OH)_2D_3$ is not affected by TGFß. Western blot analysis (A) was performed with nuclear proteins from ROS 17/2.8 cells, which were separated in a 12% SDS-PAGE gel and transferred to a solid support. Detection of VDR levels was performed using the VDR antibody IVG8C11. The abbreviations used are the same as indicated in Figs. 5 and 6, and the VDR protein (V) is indicated by the arrowhead. The subtle variations in the 1,25-$(OH)_2D_3$ treated lanes appear to reflect similar variations in the nonspecific band migrating slower than the VDR. Ligand binding assays (B) using [^{3}H]1,25-$(OH)_2D_3$ were carried out with cytosolic preparations from ROS 17/2.8 cells treated with 1,25-$(OH)_2D_3$ and/or TGFβ_2. The values on the ordinate were calculated by dividing fmol [^{3}H] 1,25$(OH)_2D_3$ bound per mg protein by the values obtained from untreated cells. Bar graphs represent mean ± SD of duplicates form two independent 1,25-$(OH)_2D_3$ ligand binding experiments. * $P<0.05$, ** $P<0.0001$ versus control.

effects of TGFß on expression of the OC and OP genes are mediated by transcriptional mechanisms. TGFβ abrogates 1,25-$(OH)_2D_3$ enhancement of OC transcription, which requires presence of the OC VDRE within the promoter. However, TGFβ also inhibits

basal OC transcription in the absence of 1,25-$(OH)_2D_3$, which occurs irrespective of the presence of VDRE sequences. TGFβ repression of basal OC gene transcription is mediated by a TGFβ response element (TGRE) in the proximal promoter of the OC gene (47). Transcriptional repression involves binding of AP-1 family members to the TGRE (47). Our results show that TGFβ increases AP-1 binding activity both in the presence or absence of 1,25-$(OH)_2D_3$ in ROS 17/2.8 cells. Thus, increased levels of AP-1 binding may contribute to inhibition of OC gene transcription.

We note that TGFβ also inhibits basal OP gene transcription. This inhibition of OP gene transcription, as monitored by reporter gene expression following treatment with TGFβ for 24 h, is not reflected by changes in OP mRNA levels. It appears that TGFβ dependent inhibition of OP transcription may be compensated by post-transcriptional modulatory events, including mRNA processing, transport, and/or stability. Alternatively, the observed differences between TGFß effects on OP transcription and OP mRNA level may reflect differences in the regulation of the endogenous gene and the OP CAT chimeric reporter gene construct. Noda *et al.* (16) observed that TGFß increases OP mRNA levels, concomitant with stimulation of OP gene transcription, based on results from nuclear run-on assays following a 72 hr treatment with TGFβ. This apparent discrepancy in the observed effects of TGFβ on OP gene expression may relate to differences in experimental design, as well as perhaps reflect chronic effects of TGFβ on cell growth and differentiation of osteoblasts.

We tested the hypothesis that transcriptional repression of 1,25-$(OH)_2D_3$-enhanced OC and OP gene expression may be achieved by modulating protein/DNA interactions at the OC and OP VDREs. Transcriptional up-regulation of the OC gene by 1,25$(OH)_2D_3$ requires the positive cis-acting VDRE, which interacts with VDR/RXR heterodimers *in vitro* (49, 54-57) and *in vivo* (58). Our results show that TGFβ inhibits the 1,25-$(OH)_2D_3$ enhancement of OC VDRE-mediated transcription and the 1,25-$(OH)_2D_3$-dependent induction of VDR containing transcription factor complexes interacting with the OC- and OP-VDREs. Thus, we propose that TGFβ may directly influence OC and OP gene transcription by modulating 1,25$(OH)_2D_3$-dependent protein/DNA interactions at the OC and OP VDREs. This concept is supported by previous findings that another polypeptide regulator of OC transcription, TNF-α (59), influences 1,25$(OH)_2D_3$ regulated transcription by modifications of protein/DNA interactions at the VDRE.

Regulation of the nuclear representation of VDR/RXR heterodimers may occur at multiple levels of control. For example, our results show that VDR mRNA levels are

increased in a 1,25-$(OH)_2D_3$-dependent manner, which is reflected by increased VDR protein levels as monitored by Western blot analysis and 1,25-$(OH)_2D_3$ ligand binding assays, as well as induction of VDR/RXR heterodimer interactions with the VDRE. In coincubation experiments with 1,25-$(OH)_2D_3$, TGFß reduces the 1,25-$(OH)_2D_3$-dependent increase in VDR mRNA, but this decrease is not paralleled by a reduction in VDR protein levels. This finding is consistent with the observation that stabilization of VDR protein by 1,25-$(OH)_2D_3$ ligand binding, contributes to the homologous up-regulation of the VDR (60). Taken together, these data suggest that VDR mRNA levels are not rate-limiting for synthesis of VDR protein, and VDR protein levels are not rate-limiting for formation of VDR/RXR heterodimers in cells treated with both TGFβ and 1,25-$(OH)_2D_3$. Thus, the mechanism by which TGFß reduces VDR/RXR heterodimer interactions at VDREs appears to involve post-translational events, *e.g.* phosphorylation and/or protein/protein interactions, including effects of TGFß on nuclear availability of RXR proteins.

Phosphorylation of the VDR influences VDRE binding activity and transcriptional activation function of VDR/RXR heterodimers (40, 50, 61, 62). Hyper-phosphorylation of the VDR correlates with decreased VDRE binding activity (50, 61) and phosphorylation pathways involving protein kinase C may post-translationally modify VDR *in vivo*. Because TGFβ-mediated signal transduction occurs via protein kinase C (63) and 1,25-$(OH)_2D_3$ action also involves protein kinase C (61, 64), the possibility arises that TGFß may alter phosphorylation of the VDR with consequential repression of VDR/RXR binding activity and transcriptional activation.

Accumulating evidence suggests that the functions of 1,25-$(OH)_2D_3$ and TGFß in the regulation of bone metabolism and parameters of osteoblast differentiation are strongly interrelated (34, 36-38). TGFß is capable of increasing 1,25-$(OH)_2D_3$ receptor number (65, 37). 1,25-$(OH)_2D_3$ stimulates homologous up-regulation of the VDR, which is required for the induction of VDR/RXR heterodimers, resulting in the transcriptional activation of 1,25-$(OH)_2D_3$ responsive genes. 1,25-$(OH)_2D_3$ also stimulates TGFß synthesis and secretion by bone cells, as well as enhances TGFβ incorporation into bone (66, 67). The inhibitory effect of TGFß on 1,25-$(OH)_2D_3$-enhancement may provide a negative regulatory loop in the action of 1,25$(OH)_2D_3$.

The results presented here suggest that TGFβ influences OC and OP gene transcription. The effect of TGFβ on OC gene transcription may occur via a two-pronged mechanism. First, TGFβ abrogates 1,25-$(OH)_2D_3$ enhancement of OC transcription by down-regulating binding of VDR/RXR heterodimers to the OC VDRE.

Secondly, TGFβ also inhibits OC transcription in the presence or absence of 1,25-$(OH)_2D_3$, which occurs via AP-1 binding sites (47,53), perhaps mediated by TGFβ enhanced levels of AP-1 proteins. Similarities in the inhibitory effect of TGFß on 1,25-$(OH)_2D_3$-enhanced expression of two genes, OC and OP, encoding proteins with distinct properties, are consistent with functional linkage of these proteins in bone turnover.

ACKNOWLEDGMENTS

We thank Rosa Mastrototaro and Elizabeth Buffone for technical assistance, Gert-Jan van den Bemd for assistance with immunoradiometric assays, and Chaitali Banerjee and Jean Prahl for their experimental advice. We also thank Judy Rask for editorial assistance.

This work was supported by grants AR33920 and AR39588 from NIH and the Netherlands Organization for Scientific Research (Project no.900-541-131). It's contents are solely the responsibility of the authors and do not necessarily represent the official views of NIH.

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CHAPTER 4

CONSEQUENCES OF VITAMIN D RECEPTOR REGULATION FOR THE 1,25-DIHYDROXYVITAMIND_3-INDUCED 24-HYDROXYLASE ACTIVITY INOSTEOBLAST-LIKE CELLS: INITIATION OF THE C24-OXIDATION PATHWAY

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Submitted for publication

ABSTRACT

A direct relationship between vitamin D receptor (VDR) level and target cell responsiveness to 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) has been shown in osteoblast-like cell lines. However, we previously found an inverse relationship between the TGFß-induced VDR up-regulation and subsequent 1,25-$(OH)_2D_3$-induced biological responses. A clear inhibition of the 1,25-$(OH)_2D_3$-induced stimulation of osteocalcin and osteopontin expression was observed. A biological response that has formerly been shown to be coupled to VDR level is 24-hydroxylase activity. This enzyme initiates the C24-oxidation of the side-chain, followed by cleavage and ultimate metabolic clearance of both 25-$(OH)D_3$ and its metabolite 1,25-$(OH)_2D_3$. With UMR 106 (rat) and MG 63 (human) osteoblast-like cells, we show that after preincubation with TGFß, which causes an increase in VDR level, 1,25-$(OH)_2D_3$ induction of 24-hydroxylase activity is also stimulated. In addition, we provide evidence that variations in VDR level induced by other means (PTH, EGF, medium change) are also closely associated with 1,25-$(OH)_2D_3$-induced 24-hydroxylase activity. Furthermore, we show that in MG 63 cells, but not in UMR 106 cells, TGFß itself was able to increase the activity of the enzyme 24-hydroxylase. As 24-hydroxylation is the initial step in the further C24-oxidation of 1,25-$(OH)_2D_3$, our results indicate a close coupling of VDR level and the degradation of its ligand, 1,25-$(OH)_2D_3$. This mechanism may provide an important regulatory feedback in the action of 1,25-$(OH)_2D_3$ at target tissue/cell level.

INTRODUCTION

For the maintenance of calcium homeostasis regulation of the formation and degradation of the biologically active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), is important. After 25-hydroxylation of vitamin D_3 in the liver, and hydroxylation at the C1α-position in the kidney, 1α,25-$(OH)_2D_3$ is formed. Parathyroid hormone (PTH) and reduced plasma levels of calcium and phosphate are the most important stimulators of 1α-hydroxylase activity. In the kidney, 1,25-$(OH)_2D_3$ inhibits its own synthesis, but stimulates in a number of target tissues, including the kidney, the enzyme 24-hydroxylase (1). 24-Hydroxylase mediates the conversion of 1,25-$(OH)_2D_3$ to 1,24,25-$(OH)_3D_3$, which is actually the initial step in a more extensive C-24 and C-23 oxidation of the side chain (1,24,25-$(OH)_3D_3$ → 24-oxo-1,25-$(OH)_2D_3$ →

24-oxo-1,23,25-$(OH)_3D_3$) and ultimately results in the production of calcitroic acid (2,3). In other words, the self-induced metabolism of 1,25-$(OH)_2D_3$ may provide a means to regulate its concentration at the level of its target tissues (3,4).

A direct relationship between vitamin D receptor (VDR) level and target cell responsiveness (7) to 1,25-$(OH)_2D_3$, including regulation of the C24-oxidation pathway, has been shown in osteoblast-like cell lines (8-10). However, transforming growth factor-ß (TGFß), a growth factor produced by bone cells and stored in the bone matrix, which increases VDR level in osteoblast-like cells (11,12), decreases the expression of osteocalcin and osteopontin in response to 1,25-$(OH)_2D_3$ (12,13). At the moment the biological significance of the TGFß-induced increase in VDR level is unknown. In the present study, we examined the coupling of VDR level to 24-hydroxylase activity by studying the effect of TGFß-induced VDR up-regulation on subsequent 1,25-$(OH)_2D_3$ induction of the 24-hydroxylase activity.

MATERIALS AND METHODS

Materials

[23,24-^{3}H]-1,25-$(OH)_2D_3$ (120 Ci/mmol) and [26,27-^{3}H]-25-(OH)D_3 (28 Ci/mmol) were obtained from Amersham International (Aylesbury, Buckinghamshire, UK). Non-radioactive 1,25-$(OH)_2D_3$ was generously provided by LEO Pharmaceuticals BV (Weesp, The Netherlands), and TGFß type 2 by Dr. J. Feyen, Sandoz Pharma Ltd (Basel, Switzerland). Fetal calf serum (FCS), penicillin, streptomycin and L-glutamine were purchased from Flow Laboratories (Irvine, UK). αMEM medium was purchased from Sigma (St Louis, MO, USA). Bovine serum albumin (BSA) fraction 5 was obtained from ICN ImmunoBiologicals (Lisle, USA). All other reagents were of the best grade commercially available.

Cell culture

UMR 106 (rat) and MG-63 (human) osteoblast-like cells were maintained in αMEM medium supplemented with 2 mM L-glutamine, 0.1 % glucose, 100 IU/ml penicillin, 100 IU/ml streptomycin and 10% FCS at 37°C under 95% air/5% CO_2. For the experiments, cells were seeded at 40,000 cells/cm^2 in αMEM containing 10% FCS and cultured for 24 h. Next, medium was changed to αMEM medium containing 2% charcoal-treated FCS (CT-FCS) and the cells were cultured for 16 hr. To investigate the effect of TGFß, and changing the medium on VDR levels, respectively, 1 ng/ml TGFß was added to the cells cultured with 2% CT-FCS containing αMEM, or medium was changed to serum free αMEM containing 0.1% BSA. 1,25-$(OH)_2D_3$ binding assays were performed after various incubation periods. For investigation of the effects of altered receptor levels on subsequent induction of the C24-oxidation pathway by 1,25-$(OH)_2D_3$ the following incubations were chosen: 1) *pre*incubation with 1 ng/ml TGFß for 4 hr, thereby increasing VDR level, followed by addition of vehicle or several concentrations 1,25-$(OH)_2D_3$ for 1 hr, 2) no preincubation with TGFß, thereby maintaining a control VDR level, and subsequent *co*incubation of 1 ng/ml TGFß with vehicle or several concentrations of 1,25-$(OH)_2D_3$ for 1

hr (Figure 1A), 3) changing the medium to serum free αMEM containing 0.1% BSA, followed by either 0 hr (control VDR level), 3 hr (increasing VDR level) or 24 hr (decreasing VDR level) of incubation and subsequent addition of vehicle or several concentrations 1,25-$(OH)_2D_3$ for 1 hr (Figure 1B).

[^{3}H]-1,25-$(OH)_2D_3$ binding assay

1,25-$(OH)_2D_3$ ligand binding assays were performed as described by Pols et al. (6). In brief, after incubation the cells were washed with αMEM containing 2% BSA at 37°C for 2 hours to remove residual unlabelled 1,25-$(OH)_2D_3$. Then cells were extracted on ice in a hypertonic buffer consisting of 300 mM KCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM dithiothreitol, 10 mM sodium molybdate and 0.1% Triton X-100. High-speed supernatants were obtained, and 250 µl aliquots were incubated

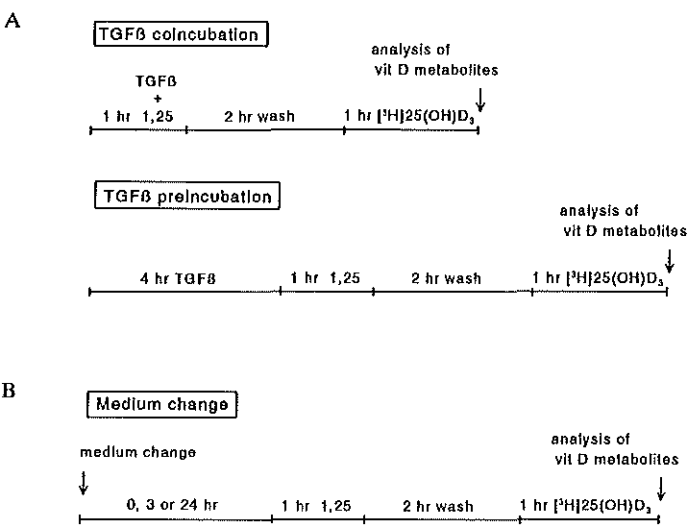

Figure 1

Incubation protocols for determining the effects of pre-and co-incubations with several factors on 1,25-$(OH)_2D_3$ induction of the C24-oxidation pathway of vitamin D_3.
Cells were plated at a density of 40,000/cm^2 and cultured for 24 hr in αMEM containing 10% FCS. Next, medium was changed to αMEM containg 2% charcoal treated FCS and cultured for 16 hr. Subsequently, A) cells were coincubated with 1 ng/ml TGFß and vehicle or several concentrations of 1,25-$(OH)_2D_3$ for 1 hr or cells were preincubated with 1 ng/ml TGFß for 4 hr followed by the addition of vehicle or several concentrations 1,25-$(OH)_2D_3$ for 1 hr, or B) medium was changed to serum free αMEM containing 0.1% BSA and cells were cultured for 0, 3 or 24 hr followed by the addition of vehicle or several concentrations 1,25-$(OH)_2D_3$ for 1 hr. After incubation with 1,25-$(OH)_2D_3$, cells were washed in αMEM containing 2% BSA at 37°C for 2 hr to remove unlabelled 1,25-$(OH)_2D_3$, followed by the incubation of 10^{-8} M [^{3}H]-25-$(OH)D_3$ in αMEM containing 0.1% BSA for 1 hr. Next, the conversion of [^{3}H]-25-$(OH)D_3$ to [^{3}H]-24,25-$(OH)_2D_3$ was determined by HPLC.

overnight at 4°C for single point assays with 0.25 nM [^{3}H]-1,25-$(OH)_2D_3$ in the absence or presence of a 200-fold molar excess of unlabelled hormone. After removal of the unbound ligand, receptor-bound 1,25-$(OH)_2D_3$ was quantitated by liquid scintillation counting.

24-Hydroxylase assay and detection of vitamin D metabolites

Induction of the C24-oxidation pathway and 24-hydroxylase activity was determined by measuring the reduction in substrate ([^{3}H]-25-$(OH)D_3$) and/or the appearance of [^{3}]-24,25-$(OH)_2D_3$ as described previously (6). At the end of the incubation period, as described in cell culture, medium was removed and cells were washed with αMEM containing 2% BSA at 37°C for 2 hours to remove residual unlabeled 1,25-$(OH)_2D_3$ and subsequently incubated with 10^{-8} M [26,27-^{3}H]-25-$(OH)D_3$ for 1 hour. Next, medium was collected, cells were scraped in PBS-Triton X-100 and pooled with the medium. Samples were extracted three times with diethylether. The ether-extractable fraction was subjected to high pressure liquid chromatography (HPLC). Injector, pumps and uv detector of the HPLC system were purchased from Millipore Waters (Milford, MA, USA). HPLC analysis was performed using either two 0.3 x 10 cm SI columns (Chrompack, Bergen op Zoom, The Netherlands) with hexane : isopropanol : methanol (96:3.2:0.8) as the solvent at a flow rate of 0.4 ml/min or two 0.3 x 10 cm Spherisorb 5 CN columns (Chrompack, Bergen op Zoom, The Netherlands) with hexane:isopropanol:methanol (94:5:1) as the solvent at a flow rate of 0.6 ml/min. The radioactive vitamin D_3 metabolites were identified by their retention time after calibration with standard 25-$(OH)D_3$ and 24,25-$(OH)_2D_3$.

DNA and protein measurements

Changes in DNA content were assessed by the fluorimetrical method of Johnson-Wint and Hollis (14). Protein concentration was measured according to the method of Bradford (15).

RESULTS

The effects of TGFß and medium change on vitamin D receptor level

Figure 2 illustrates the time-course of the 1 ng/ml TGFß effect on VDR level in UMR 106 (Figure 2A) and MG 63 (Figure 2B) cells. In both cell lines TGFß has a maximal effect after4-6 hr of incubation, although a significant increase was already observed after 2 hr in UMR 106 and 4 hr in MG 63 cells. Figure 2C shows the effect of a medium change from αMEM containing 2% CT-FCS to serum free αMEM containing 0.1 % BSA. A doubling of VDR content was observed 3 hr after medium change, followed by a decline to levels significantly below control level at 24 hr. The up-regulation of VDR level was blocked by the addition of actinomycin D and cycloheximide (data not shown), indicating that the observed increase is due to increased VDR transcription and new protein synthesis. Finally, under all conditions tested no change in receptor affinity for 1,25-$(OH)_2D_3$ was observed (data not shown).

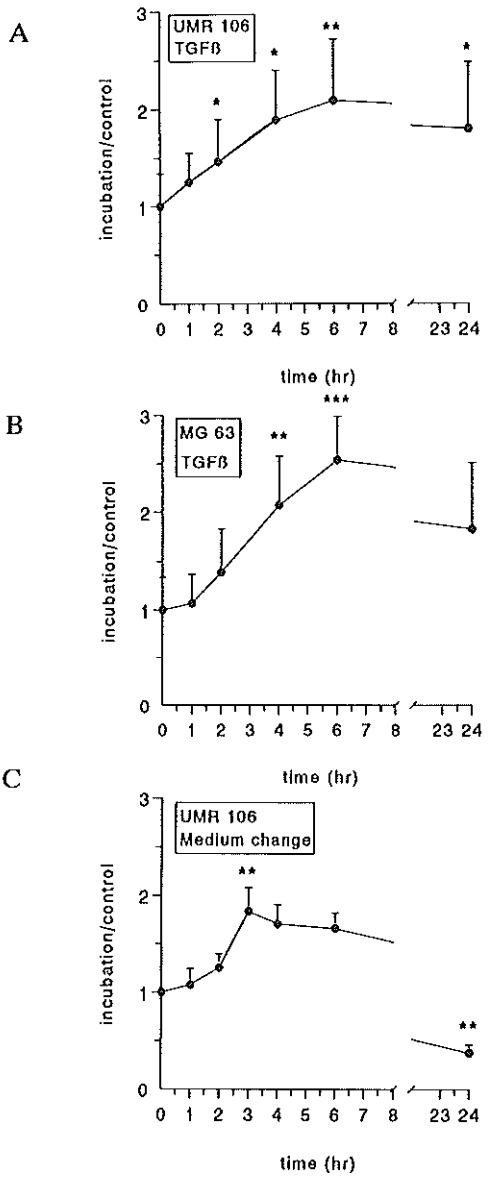

Figure 2

Time course of the modulation of VDR level by TGFß treatment and medium change.
A) UMR 106 and B) MG 63 cells were incubated with 1 ng/ml TGFß for several incubation periods and VDR levels were determined by 1,25-$(OH)_2D_3$ binding assay. C) Medium of UMR 106 cells was changed from 2% charcoal-treated FCS to serum free αMEM containing 0.1% BSA and after several incubation periods VDR levels were determined by 1,25-$(OH)_2D_3$ binding assay. Data are expressed as treatment/control ± SD as determined by single point assays in duplicate of two separate experiments. * $p<0.05$, ** $p<0.005$, *** $p<0.001$ versus control.

The effect of TGFß pre- and co-incubation on 1,25-$(OH)_2D_3$-induced 24-hydroxylase activity

The significance of TGFß-induced VDR up-regulation for 1,25-$(OH)_2D_3$ induction of 24-hydroxylase activity was studied by preincubating UMR 106 and MG 63 cells with 1 ng/ml TGFß for 4 hr, causing an up-regulation of VDR level, followed by the addition of several concentrations 1,25-$(OH)_2D_3$ (Figure 1A). To prevent interference of homologous up-regulation of the VDR, which is the increase in VDR level induced by 1,25-$(OH)_2D_3$, the incubations with 1,25-$(OH)_2D_3$ were limited to 1 hr. Induction of the 24-hydroxylase activity was measured by HPLC analysis as the conversion of [^{3}H]-25-$(OH)D_3$ to [^{3}H]-24,25-$(OH)_2D_3$ during 1 hr of incubation. Prior to incubation with labelled 25-$(OH)D_3$, cell cultures were washed at 37°C for 2 hr in serum free medium containing 2% BSA to remove residual unlabelled 1,25-$(OH)_2D_3$ (Figure 1A). For comparison, experiments were performed in which TGFß was coincubated with 1,25-$(OH)_2D_3$ for 1 hr. TGFß incubation for 1 hr does not significantly affect VDR content in UMR 106 and MG 63 cells (Figures 2A and B).

Figures 3 and 4 illustrate the effects of pre- and co-incubation with TGFß on 1,25-$(OH)_2D_3$-induced C24-oxidation in UMR 106 and MG 63 cells, respectively. Preincubation with 1 ng/ml TGFß strongly enhances the 1,25-$(OH)_2D_3$-induced conversion of 25-$(OH)D_3$ to 24,25-$(OH)_2D_3$ (Figure 3A), concomitant with its stimulation of VDR level in UMR 106 cells (Figure 2). Also in MG 63 cells, preincubation with TGFß resulted in increased induction of 24-hydroxylase activity (Figure 4A). However, in contrast to UMR 106 cells, it is not clear whether this increase is fully related to an increased VDR level, because a 4 hr preincubation with TGFß, followed by a control incubation (i.e. without 1,25-$(OH)_2D_3$) also induced 24,25-$(OH)_2D_3$ accumulation (Figure 4A). As to the difference in effects of TGFß in UMR 106 and MG 63 cells (c.f. Figures 3A and 4A), the possibility exists that 24,25-$(OH)_2D_3$ is already converted to subsequent metabolites in the C24-oxidation pathway in MG 63 cells. Therefore, after incubation with TGFß and 10^{-8} M 1,25-$(OH)_2D_3$ in MG 63 cells, we also examined the loss of the [^{3}H]-25-$(OH)D_3$ substrate, which reflects the overall conversion into "catabolic" metabolites. Figure 5 shows that preincubation with TGFß results in an greater loss in [^{3}H]-25-$(OH)D_3$ substrate than control incubation. This observation indicates that additional metabolites have been formed. But more importantly it suggests that a relationship exists between TGFß-regulated VDR level and the induction of the C24-oxidation pathway in MG 63 cells.

Coincubation with TGFß did not significantly affect the induction of 24-

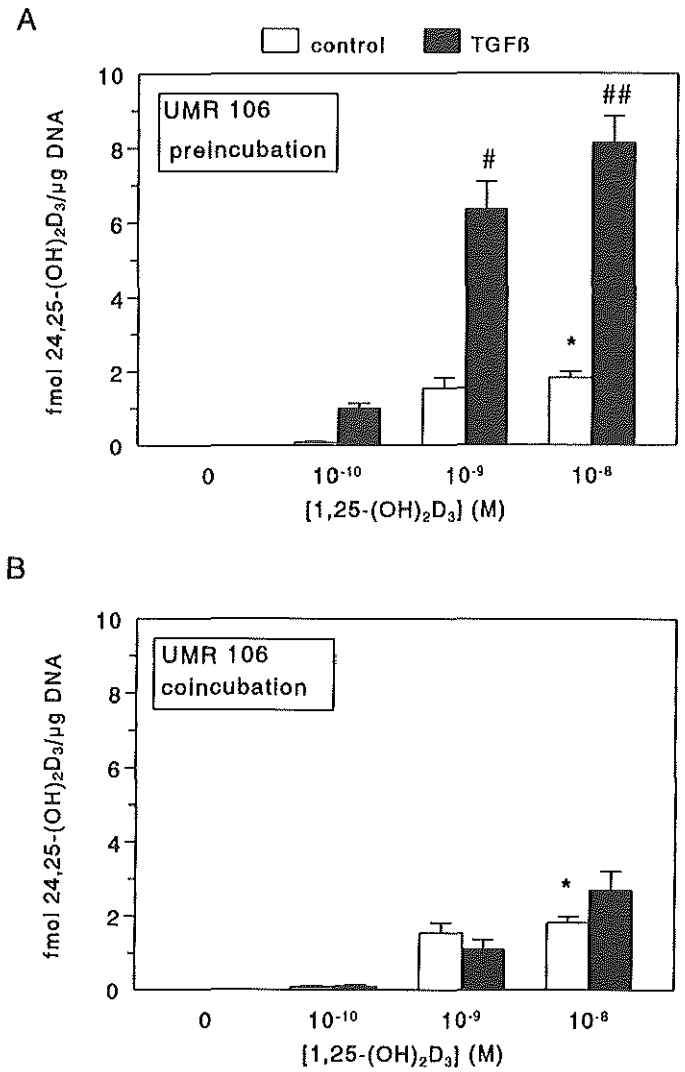

Figure 3

Preincubation, but not coincubation, with TGFß enhances the induction of 24-hydroxylase activity by 1,25-$(OH)_2D_3$ in UMR 106 cells.

UMR 106 cells were preincubated for 4 hr with 1 ng/ml TGFß followed by the addition of vehicle or 1,25-$(OH)_2D_3$ for 1 hr or coincubated with 1 ng/ml TGFß and vehicle or 1,25-$(OH)_2D_3$ for 1 hr as described in Figure 1A and the effect on C24-oxidation was determined by measuring the conversion of [^{3}H]-25-$(OH)D_3$ to [^{3}H]-24,25-$(OH)_2D_3$. A) The effect of preincubation with TGFß on the induction of C24-oxidation by 1,25-$(OH)_2D_3$. B) The effect of coincubation of TGFß and 1,25-$(OH)_2D_3$ on C24-oxidation. * $p<0.05$ versus control. # $p<0.01$, ## $p<0.005$ calculated as significance of interaction.

hydroxylase by 1,25-$(OH)_2D_3$ in UMR 106 and MG 63 cells (Figures 3B and 4B). In MG 63 cells, we show that coincubation of TGFß and 1,25-$(OH)_2D_3$ also did not affect the loss of [^{3}H]-25-$(OH)D_3$ substrate (Figure 5). Incubation of MG 63 cells with TGFß alone for 1 hr resulted in a small induction of 24-hydroxylase activity (Figure 4B).

The effects of other mediators on VDR level and their consequences for the induction of the C24-oxidation pathway by 1,25-$(OH)_2D_3$

In order to address the relationship between VDR level and the C24-oxidation pathway more extensively, we examined the effect of changes in VDR by other means than TGFß incubation on the induction of the C24-oxidation pathway. Preincubation for 4 hr with 10 nM PTH, which has previously been shown to increase VDR level after 4 hr of incubation in UMR 106 cells (17,18), also results in increased induction of the C24-oxidation pathway by 1,25-$(OH)_2D_3$ in UMR 106 cells (data not shown).

Another way to modulate VDR number in UMR 106 cells is changing cell culture medium from CT-FCS to serum free (16). In this way we could time-dependently either increase (3 hr) or decrease (24 hr) the VDR level (Figure 2C). The mechanism of and the factors involved in the medium change-induced modulation of the VDR are unknown. However, it provides an excellent tool to examine by one type of manipulation the significance of either an increased or decreased VDR level. Therefore, medium was changed from 2% CT-FCS αMEM to serum free αMEM containing 0.1% BSA and after 0, 3, or 24 hr of incubation in this medium 1,25-$(OH)_2D_3$ was added for 1 hr. Subsequently, cells were washed for 2 hr in MEM containing 2% BSA, followed by the incubation with 10^{-8} M [^{3}H]-25$(OH)D_3$ for 1 hr (Figure 1C). Figure 6 shows a dose-dependent effect of 1,25-$(OH)_2D_3$ on the loss of [^{3}H]-25-$(OH)D_3$ substrate. The increased receptor level at 3 hr after medium change is parallelled by an increased catabolism. Comparably, decreased receptor level at 24 hr after medium change is parallelled by a reduced catabolism.

In addition, we found that preincubation for 24 hr with 10 ng/ml epidermal growth factor (EGF), a growth factor which has previously been shown to decrease VDR level after 24 hr of incubation in the osteoblast-like cell line UMR 106 (19), results in a decreased induction of the C24-oxidation pathway by 1,25-$(OH)_2D_3$ in UMR 106 cells (data not shown).

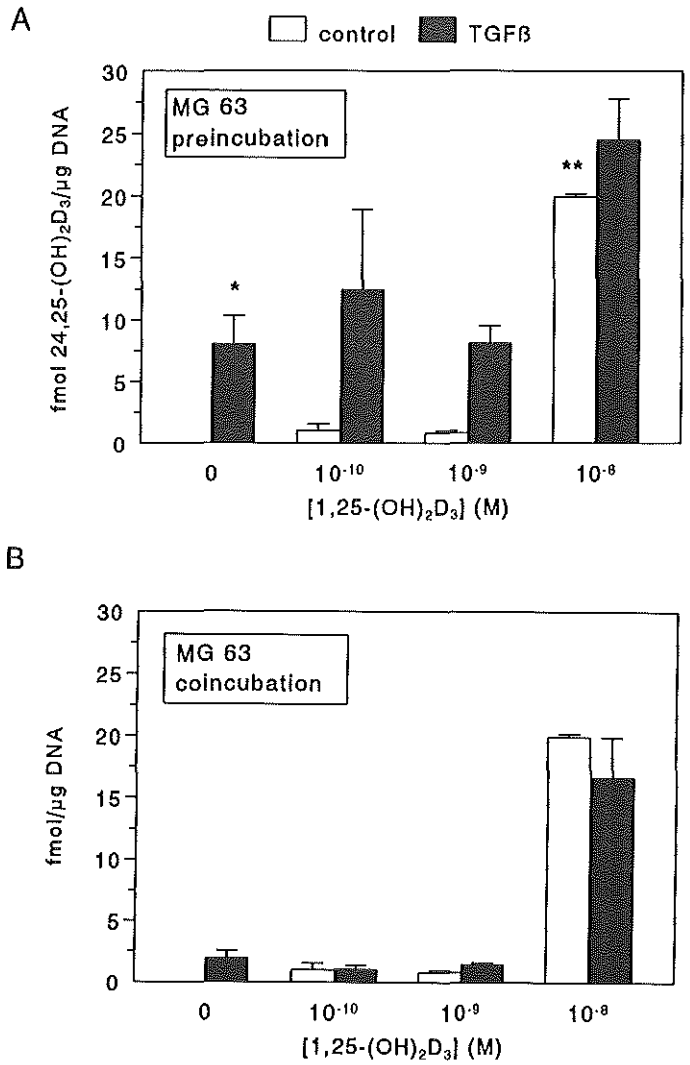

Figure 4

The effects of pre- and coincubation with TGFß in the absence or presence of 1,25-$(OH)_2D_3$ on 24-hydroxylase activity in MG 63 cells.

MG 63 cells were either preincubated with 1 ng/ml TGFß for 4 hr followed by the addition of vehicle or 1,25-$(OH)_2D_3$, or cells were coincubated with 1 ng/ml TGFß and vehicle or several concentrations of 1,25-$(OH)_2D_3$ for 1 hr as described in Figure 1A. The effect on C24-oxidation was determined by measuring the conversion of [^{3}H]-25-$(OH)D_3$ to [^{3}H]-24,25-$(OH)_2D_3$. A) The effect on 1,25-$(OH)_2D_3$-induced formation of 24,25-$(OH)_2D_3$ by preincubation with TGFß for 4 hr. B) The effect of 1,25-$(OH)_2D_3$ -induced formation of 24,25-$(OH)_2D_3$ after coincubation with TGFß for 1 hr. * $p<0.05$, ** $p<0.01$ versus control.

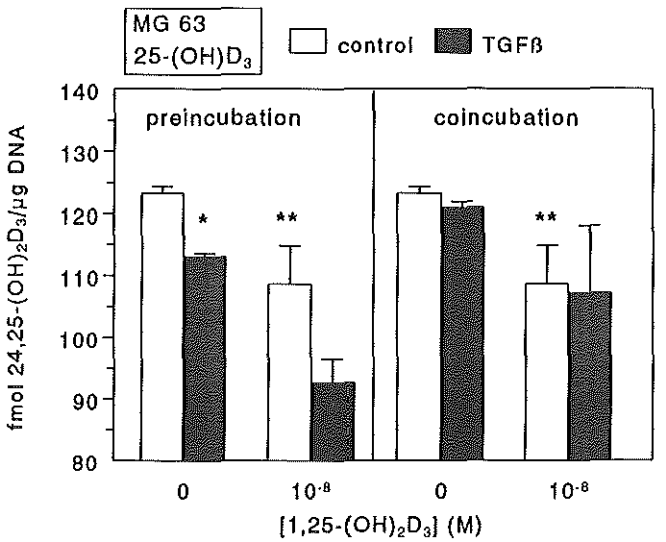

Figure 5

The effects of pre- and coincubation with TGFß on the 1,25-$(OH)_2D_3$-induced C24-oxidation pathway. MG 63 cells were either preincubated with 1 ng/ml TGFß for 4 hr followed by the addition of vehicle or 1,25-$(OH)_2D_3$, or cells were coincubated with 1 ng/ml TGFß and vehicle or 10^{-8} M 1,25-$(OH)_2D_3$ for 1 hr as described in Figure 1A. The effect on C24-oxidation was determined by measuring the loss of [^{3}H]-25-$(OH)D_3$ substrate. * $p<0.05$, ** $p<0.01$ versus control.

DISCUSSION

In general, the present study provides evidence for a close relationship between VDR regulation and the induction of 24-hydroxylase activity by 1,25-$(OH)_2D_3$. Previous experiments in our laboratory have clearly indicated that 24-hydroxylation only represents the initial step of the C24-oxidation pathway. Furthermore, the present results underline the biological relevance of the TGFß-induced increase in VDR level, which has previously been shown not to be related to two other responses to 1,25-$(OH)_2D_3$, osteocalcin and osteopontin expression (12,13). The observed relationship between VDR up-regulation and a subsequent higher 1,25-$(OH)_2D_3$-induced 24-hydroxylase activity is not unique for TGFß. We show that medium change- and PTH-induced (18) VDR up-regulation as well as medium change- and EGF-induced (19) down-regulation of the VDR is followed by enhanced

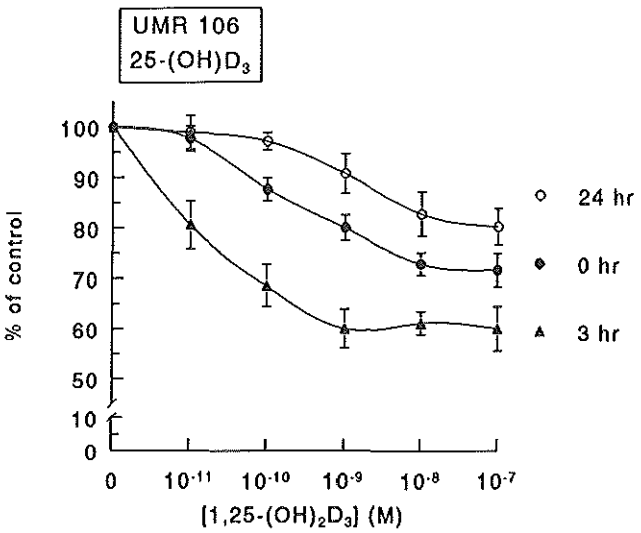

Figure 6

The effects of medium change on the 1,25-$(OH)_2D_3$ induction of the C24-oxidation pathway as measured by the loss of [^{3}H]-25-$(OH)D_3$ substrate.
Medium of UMR 106 cells was changed to serum free αMEM containing 0.1% BSA and cells were cultured for 0, 3 or 24 hr followed by the induction of C24-oxidation by several concentrations of 1,25-$(OH)_2D_3$ for 1 hr as described in Figure 1B. Loss of [^{3}H]-25-$(OH)D_3$ was calculated from pmol [^{3}H]-25-$(OH)D_3/10^6$ cells.

or reduced 1,25-$(OH)_2D_3$ induction of 24-hydroxylase activity, respectively. These observations fit with data obtained by others. Armbrecht et al. (20) also demonstrated a synergistic increase in 24-hydroxylase activity after combined treatment of PTH and 1,25-$(OH)_2D_3$ in osteoblast-like cells. Despite species differences with respect to the response to retinoic acid in VDR regulation, a close parallel was observed between the regulation of VDR level and the self-induced catabolism of 1,25-$(OH)_2D_3$ in rat and mouse osteoblast-like cells (8). Dexamethasone induces an increase in VDR level and concomitantly increases 1,25-$(OH)_2D_3$ induction of 24-hydroxylase activity (9). Proliferation-related variances in VDR level in mouse osteoblast-like cells were also coupled to the induction of 24-hydroxylase activity by 1,25-$(OH)_2D_3$ (11). Also in other tissues, VDR mRNA and 24-hydroxylase mRNA expression are concomitantly induced by 1,25-$(OH)_2D_3$, which supports the relevance of the VDR for 24-hydroxylase activity (21-27).

In our experiments, incubation with 1,25-$(OH)_2D_3$ for 1 hr followed by a 2 hr period of washing the cells with αMEM containing 2% BSA was enough to induce the 24-hydroxylase activity. It remains unclear, whether the effect of 1,25-$(OH)_2D_3$ on 24-hydroxylase in this short period is caused by enhancement of the expression of the gene or by enhancement of enzyme activity. Induction of gene transcription is a possibility, because two vitamin D responsive elements have been identified in the promoter of the 24-hydroxylase gene, which are involved in the transcriptional regulation of 24-hydroxylase by 1,25-$(OH)_2D_3$ (28-31). However, the time needed for the induction of 24-hydroxylase mRNA varies among cell lines and tissues from 1 hr (32) to 24 hr (33).

An interesting observation in the present study is that in MG 63 cells TGFß incubation alone appeared to induce 24-hydroxylase activity. MG-63 cells may, unlike UMR 106 cells, contain the mediator(s) to induce C24-oxidation by TGFß. Induction of 24-hydroxylase activity by 1,25-$(OH)_2D_3$ has previously been shown to be partly mediated through protein kinase C in intestinal epithelial cells (34) and in renal cells (35). Furthermore, TGFß responses have also been reported to be mediated by protein kinase C (36-38). However, protein kinase C activity was not affected by TGFß in UMR 106 cells (39). Thus, the possibility arises that in MG 63 cells TGFß induction of 24-hydroxylase activity may be mediated by protein kinase C. Despite the lack of knowledge on this mechanism, induction by TGFß results in an enhanced 24-hydroxylase activity and thereby an increased degradation of biologically active 1,25-$(OH)_2D_3$. We extrapolate the data obtained with labelled 25-$(OH)D_3$ to 1,25-$(OH)_2D_3$, because up to now 24-hydroxylase is considered the initial step in the C24-oxidation pathway of both 1,25-$(OH)_2D_3$ and 25-$(OH)D_3$.

In conclusion, we and others have provided evidence for the coupling of VDR level to the magnitude of induction of the C24-oxidation pathway by 1,25-$(OH)_2D_3$ in osteoblast-like cells. This strict coupling does not exist for several other bioresponses to 1,25-$(OH)_2D_3$, including osteocalcin, osteopontin (12,13) and inhibition of collagen synthesis (40), indicating that additional mechanisms compensate or overrule the effect of changed receptor level in the response to 1,25-$(OH)_2D_3$. The coupling of VDR level to the catabolic pathway of vitamin D_3 may provide an important negative feedback mechanism in the regulation of biological responses to 1,25-$(OH)_2D_3$ at target cell level.

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CHAPTER 5

GROWTH FACTOR-SPECIFIC REGULATION OF 1,25-DIHYDROXYVITAMIN D_3-STIMULATED OSTEOCALCIN AND OSTEOPONTIN EXPRESSION IN OSTEOBLAST-LIKE CELLS

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ABSTRACT

Both locally produced factors and systemic factors are involved in the regulation of bone metabolism. Systemic factors have been shown to modulate the production of local factors, thereby regulating bone formation and resorption. However, the presence of locally produced factors may also have implications for the biological response to systemic factors. Previously, we have shown that the locally produced factor transforming growth factor ß (TGFß) has an inhibitory effect on 1,25-$(OH)_2D_3$-stimulated osteocalcin and osteopontin expression after 24 hr. In this study we investigate the effects of two other locally produced growth factors, bone morphogenetic protein-2 (BMP-2) and insulin-like growth factor-I (IGF-I) on the action of 1,25-$(OH)_2D_3$ on osteocalcin and osteopontin expression in the rat osteoblast-like cell line ROS 17/2.8. In contrast to TGFß, both BMP-2 and IGF-I did not affect osteocalcin protein production and osteocalcin mRNA expression in absence or presence of 1,25-$(OH)_2D_3$ after 24 hr. BMP-2, but not IGF-I, caused an increase in 1,25-$(OH)_2D_3$-enhanced osteopontin mRNA expression. Previous experiments have shown that the inhibitory effects of TGFß on 1,25-$(OH)_2D_3$-stimulated osteocalcin and osteopontin expression were at least in part due to decreased binding of nuclear proteins to the vitamin D response elements (VDREs) in the promoter regions of these genes. In this study, like TGFß, BMP-2 appeared to decrease the 1,25-$(OH)_2D_3$-induced binding of nuclear proteins to both the osteocalcin and osteopontin VDREs. This may suggest that the BMP-2 effect on osteopontin mRNA is due to stabilization of the mRNA or that other regulatory sites in the promoters of osteocalcin and osteopontin play a more prominent role in the regulation of gene transcription by BMP-2. IGF-I did not affect binding of nuclear proteins to the osteocalcin VDRE and slightly reduced binding of nuclear proteins to the osteopontin VDRE. In conclusion, this study shows growth factor-specific regulation of 1,25-$(OH)_2D_3$-stimulated osteocalcin and osteopontin expression in the ROS 17/2.8 osteoblast-like cell line.

INTRODUCTION

In bone, several factors are locally produced and are present either in soluble form or bound to the bone matrix. Among the matrix bound factors are transforming growth factor ß (TGFß), bone morphogenetic protein-2 (BMP-2) and insulin-like

growth factor I (IGF-I). TGFß and BMP-2 belong to the TGFß superfamily and are dimers of 25 kD and 30 kD, respectively. TGFß is synthesized and secreted in a latent form, which can be activated by the low pH under the ruffled border of the osteoclast (1,2). BMP-2 is synthesized as a precursor molecule, which is posttranslationally modified before the active dimer is secreted (3). Both factors enhance bone formation *in vivo*, although only BMP-2 is able to induce ectopic bone formation (3,4). IGF-I is a single-chain polypeptide, which is also known to affect bone metabolism (5).

The seco-steroid hormone 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) is an important regulator of calcium homeostasis and bone remodeling (6). 1,25-$(OH)_2D_3$ exerts its actions primarily through the vitamin D receptor (VDR). The VDR belongs to the steroid/retinoid/thyroid hormone receptor family and acts by binding to distinct vitamin D response elements (VDREs) in the promoter regions of vitamin D responsive genes, including the rat (7-9) and human (10,11) osteocalcin, the mouse osteopontin (12), rat 25-hydroxy- and 1,25-dihydroxy-24-hydroxylases (13-16), calbindin D28k (17), ß integrin (18) and carbonic anhydrase II (19).

Osteocalcin and osteopontin are two major noncollagenous bone matrix proteins, which are highly expressed with the onset of matrix mineralization during the *in vitro* formation of bone-like tissue by normal diploid rat osteoblasts (20). However, in ROS 17/2.8 osteoblast-like cells, both proteins are constitutively expressed. The precise function of osteocalcin and osteopontin is unknown, but they have been proposed to play a role in bone resorption and/or mineralization (21-27).

Systemic hormones play a role in the regulation of the production of local factors by skeletal cells and it has been suggested that local factors are the mediators by which systemic factors exert their effects on bone. However, the presence of local factors may also influence the effect of systemic hormones on skeletal cells. We have previously shown that TGFß inhibits the 1,25-$(OH)_2D_3$-enhanced expression of both osteocalcin and osteopontin (28,29). Furthermore, differential effects of TGFß, BMP-2 and IGF-I have been found in 1,25-$(OH)_2D_3$- and PTH-stimulated bone resorption (Chapter 6). In this study, we investigated the significance of the local factors BMP-2 and IGF-I, as compared to TGFß, for the action of 1,25-$(OH)_2D_3$ on osteocalcin and osteopontin expression in ROS 17/2.8 osteoblast-like cells.

MATERIALS AND METHODS

Materials

[23,24-^{3}H]-1,25$(OH)_2D_3$ (105.5 Ci/mmol) was obtained from Amersham International (Aylesbury, Buckinghamshire, United Kingdom) and non-radioactive 1,25$(OH)_2D_3$ was generously provided by LEO Pharmaceuticals BV (Weesp, the Netherlands). TGFß type 2 was a generous gift of Dr. J. Feyen, Sandoz Pharma Ltd (Basel, Switzerland) and BMP-2 of Genetics Institute (Cambridge, MA, USA). IGF-I was purchased from GroPep (Adelaide, Australia). Fetal calf serum (FCS), penicillin, streptomycin and glutamine were purchased from Flow Laboratories (Irvine, United Kingdom), and αMEM medium from Sigma (St Louis, MO, USA). The rat osteocalcin probe (0.52 kb) and the rat osteopontin probe (1.3 kb) were generously provided by Dr. M. Noda (West Point, PA, USA) and rat osteocalcin antiserum by Prof. R. Bouillon (Leuven, Belgium). All other reagents were of the best grade commercially available.

Cell culture

ROS 17/2.8 cells were a gift from Dr. S.B. Rodan (Merck Sharp & Dohme Research Laboratories, Westpoint, PA, USA). For experiments, cells were seeded at 50,000 cells/cm^2 and cultured for 24 hr with αMEM supplemented with 2 mM glutamine, 0.1% glucose, 100 U/ml penicillin, 100 U/ml streptomycin and 10% FCS. Next, medium was replaced by αMEM with 2% charcoal-treated FCS and cells were cultured for 16 hr followed by the incubation with 1 ng/ml TGFß, 10 ng/ml BMP-2 or 10 ng/ml IGF-I in the absence or presence of 10^{-9} M or 10^{-8} M 1,25-$(OH)_2D_3$ for 24 h.

Osteocalcin assay

Osteocalcin measurements in medium of ROS 17/2.8 cells were performed according to the method described by Verhaeghe et al (30).

RNA isolation and Northern blot analysis

RNA isolation was performed according to the method of Chomszinski (31). Electrophoresis of total cytoplasmic RNA (20 μg) through a formaldehyde gel and Northern blotting (Gene Screen filters) were performed according to the method described by Davis et al (32). Northern blots were prehybridized for 2 hours at 42°C in a buffer containing 50% formamide, 0.2% SDS, 1x Denhardt's solution, 5x SSC (1x SSC = 150 mM NaCl and 15 mM Na citrate, pH 7.0), 20 mM NaH_2PO_4, 6% dextran sulphate, 1 μg/ml salmon sperm DNA and then hybridized for 16-24 h at 42°C with ^{32}P-labelled cDNA probe, either the 0.52 kb rat osteocalcin fragment or the 1.3 kb rat osteopontin fragment. Filters were washed using 2x SSC and 0.5% SDS, 1x SSC and 0.5% SDS and 0.5x SSC and 0.5% SDS wash buffers. Next, filters were exposed to X-ray film.

Gel mobility shift assay

Nuclear extracts were prepared as described by Shakoori et al (33). In brief, cells were scraped in PBS and after centrifugation the cell pellet was reconstituted in a hypotonic lysis buffer (10 mM HEPES, pH7.5, 10 mM KCl and 0.2 mM EDTA) supplemented with 0.75 mM spermidine, 0.15 mM

spermine, 1 mM EGTA, 1 mM DTT and a broad spectrum of protease inhibitors: 0.2 mM PMSF, 70 μg/ml TPCK, 10 μg/ml trypsin inhibitor, 0.5 μg/ml leupeptin and 1.0 μg/ml pepstatin. 0.5% Nonidet P40 was added and cells were lysed using a dounce homogenizer. After centrifugation nuclear extracts were obtained by incubating nuclei in a hypertonic nuclear extraction buffer (20mM HEPES, pH 7.5, 420 mM KCl, 25% glycerol and 0.2 mM EDTA) supplemented with the same reagents previously mentioned for the hypotonic lysis buffer. Desalting was performed by dilution with hypertonic nuclear extraction buffer without KCl.

For gel mobility shift assays the following oligonucleotides were synthesized:

rat osteocalcin VDRE (8): 5'-CTGCACTGGGTGAATGAGGACATTACTGA-3';

mouse osteopontin VDRE (12): 5'-ACAAGGTTCACGAGGTTCACGTCT-3';

SP-1 consensus: 5'-CGGATGGGCGGGGCCGGGGATGGGCGGGGCCGG-3'.

Gel-purified oligonucleotides were radiolabelled with [γ^{32}P] ATP as described by van Wijnen et al (34). Protein/DNA binding reactions were performed by combining 10 μl of a 5 μg protein mixture in a final KCl concentration of 100 mM with 10 μl of a DNA mixture containing 1 fmol/μl DNA, 0.2 μg/μl nonspecific competitor DNA (poly(dI-dC)*(dI-dC)) and 2 mM dithiothreitol for 15 min. Protein/DNA complexes were separated in a 4% polyacrylamide gel (acrylamide:bisacrylamide = 80:1) in 0.5x TBE electrophoresis buffer. Gels were dried and exposed to X-ray film.

RESULTS

The effects of TGFß, BMP-2 and IGF-I on 1,25-$(OH)_2D_3$ stimulation of osteocalcin production.

ROS 17/2.8 cells were incubated with 1 ng/ml TGFß, 10 ng/ml BMP-2 or 10 ng/ml IGF-I in the absence or presence of 10^{-9} M 1,25-$(OH)_2D_3$ for 24 hr. These conditions were chosen, because 1,25-$(OH)_2D_3$ has previously been shown to stimulate both osteocalcin and osteopontin expression, which was reduced by TGFß, within 24 hr and these concentrations have previously been shown to be effective in cell culture experiments (27,35,36). As is depicted in Figure 1, basal osteocalcin production was marginally reduced by TGFß, and not affected by BMP-2 and IGF-I. The 1,25-$(OH)_2D_3$ enhancement of osteocalcin production was reduced by TGFß, but not by BMP-2 and IGF-I.

The effects of TGFß, BMP-2 and IGF-I on the 1,25-$(OH)_2D_3$ induced increase in osteocalcin and osteopontin mRNA expression.

Figure 2 shows a Northern blot of the effects of 10 ng/ml IGF-I, 10 ng/ml BMP-2 and 1 ng/ml TGFß on osteocalcin and osteopontin mRNA in the absence or presence of 10^{-9} or 10^{-8} M 1,25-$(OH)_2D_3$ for 24 hr. IGF-I did not affect the 10^{-9} M 1,25-$(OH)_2D_3$ induced increase in osteocalcin and osteopontin mRNA, and slightly,

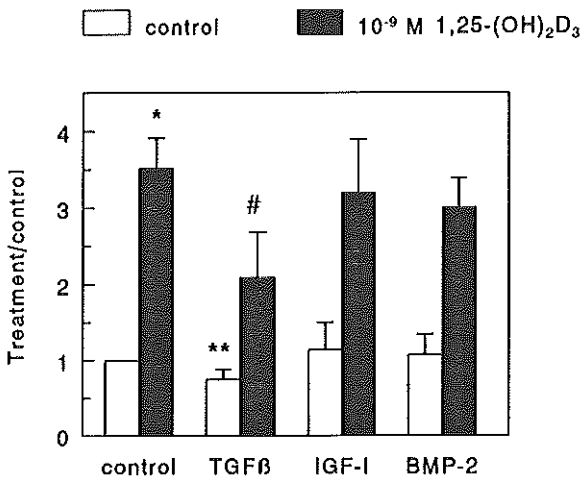

Figure 1

The effects of TGFß, BMP-2 and IGF-I on basal and 1,25-$(OH)_2D_3$-stimulated osteocalcin protein production in ROS 17/2.8 cells. ROS 17/2.8 cells were cultured as described in Materials and Methods followed by the incubation of 1 ng/ml TGFß, 10 ng/ml BMP-2 and 10 ng/ml IGF-I in the absence or presence of 10^{-9} M 1,25-$(OH)_2D_3$ for 24 hr. Osteocalcin level was measured in the medium and is depicted as treatment/control. Data are presented as mean ± SD of three independent experiments. * $p<0.001$, ** $p<0.05$ versus control. # $p<0.01$ calculated as significance of interaction.

although not significantly, enhanced the 10^{-8} M 1,25-$(OH)_2D_3$ induced increase in osteocalcin and osteopontin mRNA. BMP-2 caused an increase in 1,25-$(OH)_2D_3$ (10^{-8} M) enhancement of osteopontin mRNA, although 1,25-$(OH)_2D_3$ stimulation of osteocalcin expression was not affected. TGFß reduced the 1,25-$(OH)_2D_3$ (10^{-9} M, 10^{-8} M) induced increase in both osteocalcin and osteopontin mRNA.

The effects of TGFß, BMP-2 and IGF-I on 1,25-$(OH)_2D_3$ induction of protein/DNA interactions at the osteocalcin and osteopontin VDREs.

Because BMP-2 increased the 1,25-$(OH)_2D_3$-stimulated osteopontin expression in contrast to the inhibitory effect of TGFß, we were interested in the mechanism whereby this effect was caused. Previously, we have shown that TGFß causes at least part of its inhibitory effects on 1,25-$(OH)_2D_3$-stimulated osteocalcin and osteopontin expression by reducing the binding of nuclear protein complexes to the VDREs in the promoters of the osteocalcin and osteopontin genes. To compare the effects of BMP-

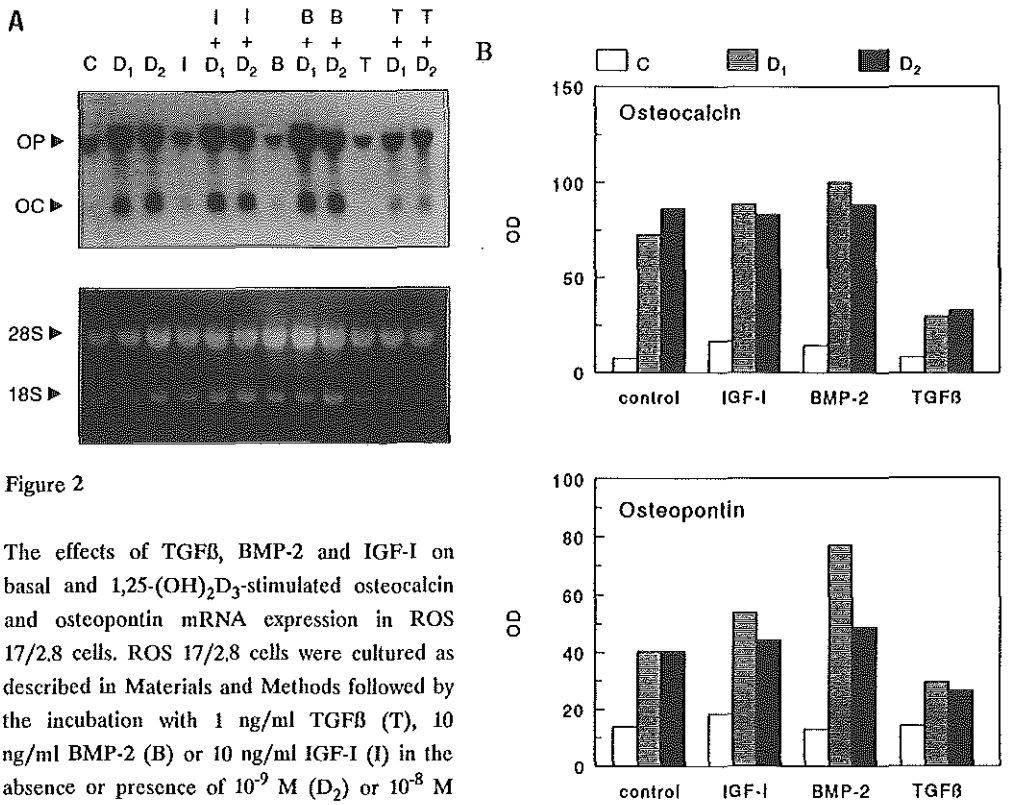

Figure 2

The effects of TGFß, BMP-2 and IGF-I on basal and 1,25-$(OH)_2D_3$-stimulated osteocalcin and osteopontin mRNA expression in ROS 17/2.8 cells. ROS 17/2.8 cells were cultured as described in Materials and Methods followed by the incubation with 1 ng/ml TGFß (T), 10 ng/ml BMP-2 (B) or 10 ng/ml IGF-I (I) in the absence or presence of 10^{-9} M (D_2) or 10^{-8} M (D_1) 1,25-$(OH)_2D_3$ for 24 hr. RNA was isolated and Northern blots were hybridized with ^{32}P-labeled rat osteocalcin (OC) and osteopontin (OP) probes. A representative Northern blot is shown in panel A. 28S and 18S RNAs, stained by ethidium bromide, represent an internal control for RNA quantitation. Panel B shows bar graphs of the optical density (OD) measurements of osteocalcin and osteopontin mRNA expression depicted in panel A.

2 and IGF-I on the binding of nuclear proteins to the osteocalcin and osteopontin VDREs with TGFß, ROS 17/2.8 cells were incubated for 24 hr with 1 ng/ml TGFß, 10 ng/ml BMP-2 and 10 ng/ml IGF-I in the absence or presence of 1,25-$(OH)_2D_3$, followed by the extraction of nuclear proteins. Subsequently, nuclear extracts were incubated with labeled oligonucleotides containing either the osteocalcin or osteopontin VDRE. No effects were observed by either growth factor on basal protein/DNA binding at both the osteocalcin and osteopontin VDRE. 1,25-$(OH)_2D_3$ induced binding of nuclear proteins to the osteocalcin and osteopontin VDRE. Like

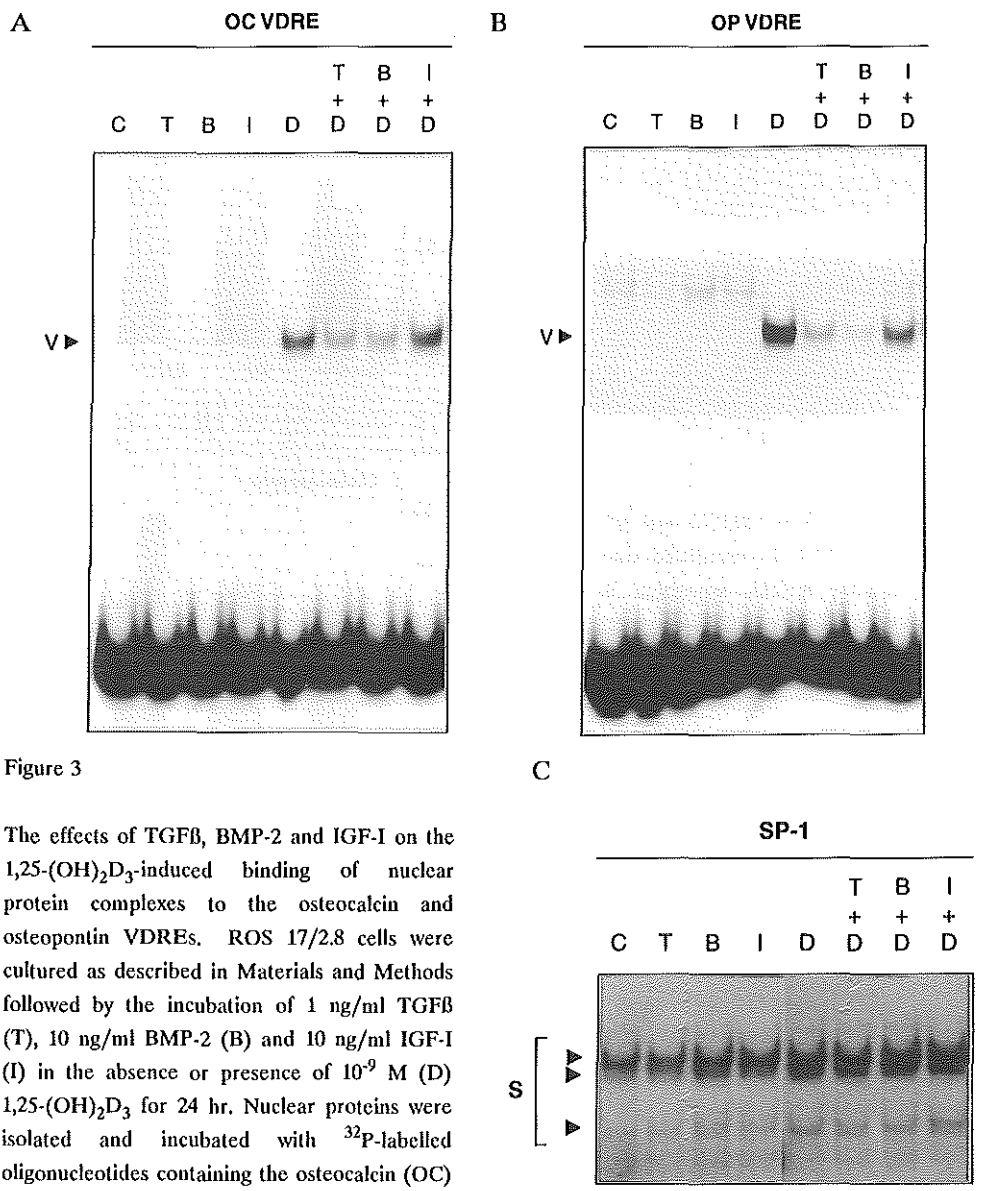

Figure 3

The effects of TGFß, BMP-2 and IGF-I on the 1,25-$(OH)_2D_3$-induced binding of nuclear protein complexes to the osteocalcin and osteopontin VDREs. ROS 17/2.8 cells were cultured as described in Materials and Methods followed by the incubation of 1 ng/ml TGFß (T), 10 ng/ml BMP-2 (B) and 10 ng/ml IGF-I (I) in the absence or presence of 10^{-9} M (D) 1,25-$(OH)_2D_3$ for 24 hr. Nuclear proteins were isolated and incubated with ^{32}P-labelled oligonucleotides containing the osteocalcin (OC) VDRE, osteopontin (OP) VDRE or SP-1 consensus. The vitamin D inducible nuclear protein complexes (V) and complexes containing nuclear proteins that specifically bind the consensus SP-1 oligonucleotide (S) are indicated by arrowheads. Binding to the SP-1 consensus oligonucleotide represents an internal control for protein quantitation.

TGFß, BMP-2 inhibited the 1,25-$(OH)_2D_3$ induced binding of nuclear proteins to the osteocalcin and osteopontin VDRE. IGF-I slightly reduced binding of nuclear proteins to the osteopontin VDRE, but did not affect binding to the osteocalcin VDRE. SP-1 oligonucleotide binding activity served as an internal control for protein quantitation.

DISCUSSION

In this study we examined the specificity of three growth factors produced by bone cells and present in the bone matrix for the rapid (24 hr) regulation of 1,25-$(OH)_2D_3$ responses in osteoblasts. As we have previously shown and confirm in the present study, within 24 hr TGFß inhibits 1,25-$(OH)_2D_3$ enhanced osteocalcin and osteopontin expression (28). 1,25-$(OH)_2D_3$-induced binding of nuclear proteins to the osteocalcin and osteopontin VDRE was reduced after TGFß incubation, which suggests that the inhibitory effects of TGFß are at least in part due to reduced binding of nuclear proteins to the VDREs in the promoter regions of these genes (29). The present study demonstrates that these effects on osteocalcin (protein/mRNA) and osteopontin (mRNA) are TGFß-specific. Previously, no differences in regulation of 1,25-$(OH)_2D_3$ action between $TGFß_1$ and $TGFß_2$ (71 % amino acid sequence homology) were observed (29). However, as we show here, another member of the TGFß superfamily, BMP-2 (35 % amino acid sequence homology with TGFß) differentially affects the action of 1,25-$(OH)_2D_3$. 1,25-$(OH)_2D_3$-induced osteocalcin production as well as osteocalcin mRNA expression were not affected by BMP-2. Yamaguchi and colleagues reported an increase in both basal and 1,25-$(OH)_2D_3$-induced osteocalcin mRNA expression in osteoblasts after 6 to 9 days of incubation with BMP-2 (35). Considering the long period of these incubations, these changes in osteocalcin expression are probably indirectly due to effects on differentiation of the osteoblasts (20,35,37). In contrast to osteocalcin, BMP-2 caused an enhancement of 1,25-$(OH)_2D_3$-induced increase in osteopontin mRNA expression. Using gelshift analysis we examined the effect of BMP-2 on 1,25-$(OH)_2D_3$-induced nuclear protein binding to the osteopontin VDRE. Surprisingly, in contrast to the enhancement of the 1,25-$(OH)_2D_3$ effect on osteopontin mRNA a very strong inhibition by BMP-2 of 1,25-$(OH)_2D_3$-induced binding of nuclear proteins to the osteopontin VDRE was observed. Also, BMP-2 strongly inhibited 1,25-$(OH)_2D_3$-induced binding of nuclear proteins to the osteocalcin VDRE, while it did not affect

1,25-$(OH)_2D_3$-induced osteocalcin mRNA and protein expression. Both the osteopontin and osteocalcin VDRE have been identified as functional elements (7-9,12). Taken together, these data demonstrate a dissociation between binding of nuclear proteins to (functional) response elements and the eventual responses at mRNA or protein level. This indicates that in the regulation by BMP-2 other mechanisms than nuclear protein binding to the VDRE may play a more prominent role, which overcome the reduced binding of nuclear proteins to the VDRE in the regulation of transcription of the osteocalcin and osteopontin genes. BMP-2 may affect binding of nuclear proteins to other regulatory sites in the promoter region of these genes or may have more prominent effects at the mRNA level. Further studies on the effects of BMP-2 on transcription of the osteocalcin and osteopontin genes will give an indication at which level osteopontin mRNA expression is regulated. At the moment the physiological significance as well as precise mechanism underlying the observed discrepancy in BMP-2 effects between nuclear protein binding to VDRE and mRNA expression induced by 1,25-$(OH)_2D_3$ remain unclear.

Data obtained with IGF-I support the growth factor specificity of the regulation of 1,25-$(OH)_2D_3$-stimulated osteocalcin and osteopontin expression. IGF-I did not affect basal and 1,25-$(OH)_2D_3$ stimulated osteocalcin protein level. IGF-I slightly enhanced the 10^{-8} M 1,25-$(OH)_2D_3$-induced increases of both osteocalcin and osteopontin mRNA, although not significantly. Previously, in human osteoblasts (38), in rat fetal calvaria (39) and in the human osteoblast-like cell line MG 63 (36) IGF-I was shown to stimulate both basal and 1,25-$(OH)_2D_3$-enhanced osteocalcin synthesis. In rat calvaria IGF-I stimulated osteocalcin synthesis after 24 hr of incubation. However, both human osteoblasts and MG 63 cells only responded to IGF-I after 48-96 hr of incubation (36,38). In our experiments, 24 hr of incubation may be too short to observe an effect of IGF-I on osteocalcin protein level, but we did observe a small stimulatory effect on 1,25-$(OH)_2D_3$-stimulated osteocalcin and osteopontin mRNA expression. Pirskanen et al reported that the effect of IGF-I on the 1,25-$(OH)_2D_3$ induced increase in osteocalcin mRNA was due to a stabilization of mRNA and not to effects on the binding of nuclear proteins to the human osteocalcin VDRE (37). Our experiments also showed that 1,25-$(OH)_2D_3$-induced binding of nuclear proteins to the osteocalcin VDRE is not affected by IGF-I. The data obtained with the osteopontin VDRE and IGF-I show, like BMP-2 although less explicit, a dissociation between effects on mRNA, which was marginally enhanced by IGF-I, and nuclear protein binding to the VDRE, which was marginally reduced by IGF-I.

Finally, the present study demonstrates specificity of interaction of TGFß, its structurally related BMP-2, and IGF-I with 1,25$(OH)_2D_3$ in osteoblasts. These data are in line with the growth factor-specific results we obtained in *in vitro* bone resorption studies (Chapter 6). Those studies show that 1,25-$(OH)_2D_3$-stimulated bone resorption is strongly inhibited by TGFß and IGF-I, whereas it is enhanced by BMP-2. Furthermore, the present study shows a dissociation between the effects of BMP-2 and IGF-I on the 1,25-$(OH)_2D_3$-induced binding of nuclear proteins to osteocalcin and osteopontin VDREs and the regulation of 1,25-$(OH)_2D_3$-stimulated osteocalcin and osteopontin mRNA expression by these factors. Additional studies to address this dissociation more specifically are in progress.

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CHAPTER 6

DIFFERENTIAL EFFECTS OF THE LOCALLY PRODUCED FACTORS TGFß, BMP-2 AND IGF-I ON THE ACTION OF SYSTEMIC FACTORS DURING IN VITRO BONE RESORPTION

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Submitted for publication

ABSTRACT

Besides systemic calciotrophic hormones, a wide variety of locally produced factors has been shown to affect bone metabolism. Therefore, it is conceivable that systemic and local factors may interfere in their actions on bone. The factors transforming growth factor ß (TGFß), bone morphogenetic protein-2 (BMP-2) and insulin-like growth factor-I (IGF-I) are produced by bone cells and are stored in the bone matrix. In the present study we investigated the effects of TGFß, BMP-2 and IGF-I on the bone resorptive action of the systemic factors 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) and parathyroid hormone (PTH). Our results show that TGFß inhibits 1,25-$(OH)_2D_3$-enhanced bone resorption, but not PTH-enhanced bone resorption, whereas IGF-I inhibits both PTH- and 1,25-$(OH)_2D_3$-enhanced bone resorption. BMP-2 has, depending on its concentration, a stimulatory effect on 1,25-$(OH)_2D_3$-enhanced bone resorption (10-100 ng/ml), or has at 1000 ng/ml a strong stimulatory effect on basal bone resorption and no further effects on 1,25-$(OH)_2D_3$ and PTH action.

In these bone resorption studies the factors were exogenously added to these cultures. Analysis of the distribution of these growth factors in the bone matrix demonstrated that in adult human bone the presence of TGFß type 2, TGFß type 3, BMP-2 and IGF-I appears to be restricted to specific areas in bone, and are not present throughout the bone. This suggests that these factors may control the response to systemic factors at particular sites in bone.

Furthermore, the effects of local and systemic factors on osteocalcin and collagenase were studied as possible mediators involved in the regulation of bone resorption. Remarkably, 1,25-$(OH)_2D_3$ has, in contrast to its stimulatory effect on bone resorption, an inhibitory effect on the osteocalcin protein levels in fetal mouse long bones. In addition, this inhibitory effect of 1,25-$(OH)_2D_3$ is also observed in the mouse osteoblast-like cell line MC3T3. 1,25-$(OH)_2D_3$ does not affect collagenase activity. In conclusion, 1,25-$(OH)_2D_3$ - and PTH-stimulated bone resorption is regulated in a growth factor-specific manner by TGFß, BMP-2 and IGF-I. This regulation is independent of effects on osteocalcin and collagenase activity. The distribution of these growth factors in bone suggests that they may control the response to systemic factors at particular sites in bone. Finally, in contrast to human and rat, in mouse 1,25-$(OH)_2D_3$ does not appear to stimulate osteocalcin production.

INTRODUCTION

The steroid hormone 1,25-$(OH)_2D_3$ and the polypeptide hormone parathyroid hormone (PTH) are important systemic regulators of calcium homeostasis and skeletal metabolism (1,2). In response to a decrease in serum calcium concentration 1,25-$(OH)_2D_3$ stimulates calcium absorption in the intestine and stimulates bone resorption. PTH increases serum calcium levels by increasing both renal calcium reabsorption and bone resorption.

In addition, also locally produced factors affect bone metabolism. Among these, members of the transforming growth factor ß (TGFß) superfamily and insulin-like growth factors (IGFs) affect osteoblast cell replication and differentiation (3). TGFß is a stimulator of bone formation, which is locally produced by osteoblasts (4) and osteoclasts (5) and is secreted and stored in an inactive form in bone (6,7). TGFß can be released from the extracellular matrix during bone resorption by osteoclasts (5) and it modulates the expression of several markers of the osteoblast phenotype (8-15). Bone morphogenetic protein-2 (BMP-2), another member of the TGFß superfamily present in the bone matrix, stimulates (ectopic) bone formation (16) and is a potent inducer of differentiation of osteoprogenitors and immature osteoblasts (17,18). The unrelated IGFs are produced by osteoblasts and stored in the bone matrix. IGF-I stimulates the proliferation and/or differentiation of osteoblasts and osteoclast progenitors (19-21). IGFs can bind to IGF-binding proteins, the IGFBPs, which may either enhance or limit the effects of IGFs (22). Data have become available indicating that systemic hormones affect the synthesis of local factors, which may be the mediators of their effects on bone metabolism (23-25). However, the presence of local factors may also regulate the eventual response to systemic hormones at target tissue/cell level (26-28).

These observations on synthesis of these growth factors by osteoblasts, their presence in bone matrix, their effects on bone cells, and their interactions with systemic regulators of bone metabolism at osteoblast level led to the following hypothesis. Locally produced growth factors present in the bone matrix and potentially released during bone resorption regulate the eventual bone resorptive response to 1,25-$(OH)_2D_3$ and PTH. This hypothesis was tested in the present *in vitro* study by examing the effect of exogenously added TGFß, BMP-2, or IGF-I on 1,25-$(OH)_2D_3$ and PTH stimulation of bone resorption. In relation to this hypothesis the distribution of these locally produced growth factors was examined in adult human

bone. In addition, in an attempt to elucidate the mechanism of the interrelated regulation of bone resorption by local and systemic factors, their effects on osteocalcin and collagenase were assessed.

MATERIALS AND METHODS

Materials

$1,25\text{-}(OH)_2D_3$ was kindly provided by LEO Pharmaceuticals BV (Weesp, The Netherlands). Rat PTH was purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). TGFß type 2 was generously provided by Dr. J. Feyen, Sandoz Pharma Ltd (Basel, Switzerland) and BMP-2 by The Genetics Institute, Cambridge, MA, USA. IGF-I and des-IGF-I were purchased from GroPep Ltd, Adelaide, Australia. ^{45}Ca was purchased from Amersham International (Aylesbury, Buckinghamshire, UK). Fetal calf serum (FCS), Biggers-Gwatkin-Judah (BGJ) medium, penicillin, streptomycin and glutamine were from Flow Laboratories (Irvine, UK). The rabbit polyclonals against human transforming growth factor-$ß_2$ and $ß_3$ ($TGFß_2$ and $TGFß_3$) were purchased from Santa Cruz Biotechnology, California, USA and against human insulin-like growth factor-I (IGF-I) was obtained from GroPep, Adelaide, Australia. The mouse monoclonal antibody h3b2/17.8.1 against BMP-2 was kindly provided by The Genetics Institute, Cambridge, MA, USA. All other reagents were of the best grade commercially available.

Bone resorption assay

The effects of systemic and local factors on bone resorption were assessed by measuring the percentage of ^{45}Ca release from prelabelled 17-day old fetal mouse radii and ulnae after 3 and 6 days of culture which is based on the method described by Raisz (29). Mice were injected subcutaneously with 30 μCi of ^{45}Ca on day 16 of gestation. Fetal mice were removed on day 17 and labeled radii/ulnae were dissected. Subsequently, radii/ulnae were cultured in 400 μl of BGJ medium with 5% charcoal-treated FCS at 37°C for 24 hr to reduce free exchangeable calcium. Then medium was refreshed and cells were incubated with or without systemic factors ($1,25\text{-}(OH)_2D_3$ and PTH) in the absence or presence of growth factors (TGFß, BMP-2 and IGF-I). After 3 days medium was removed and replaced by medium containing the same test compound(s). After another 3 days medium was removed and radii/ulnae were decalcified with 5% formic acid. The amount of ^{45}Ca was determined in medium collected after 3 and 6 days, as well as in the 5% formic acid extracts using a liquid scintillation counter and used to calculate the total ^{45}Ca content and the cumulative amount of ^{45}Ca released from radii/ulnae.

Mouse osteocalcin assay

Osteocalcin levels were measured in medium and extracts of fetal mouse long bone cultures and the osteoblast-like cell line MC3T3 according to the method of Prof R. Bouillon (personal communication; 30). Extracts of fetal mouse long bones were prepared either by the addition of 5% formic acid to radii/ulnae for 24 hr, sonification and neutralization (pH 7.4) by the addition of NaOH or by the

addition of a 6 M guanidine-HCl/0.1 M Tris-buffer (pH 8.0) followed by sonification. MC3T3 cells were cultured in αMEM containing 10% FCS for 2 weeks till confluency and were subsequently incubated with 1,25-$(OH)_2D_3$ for 48 hr. MC3T3 cells were extracted by the addition of a 6 M guanidine-HCl/0.1 M Tris-buffer (pH 8.0) followed by sonification.

Extraction of fetal mouse long bones for collagenase assay

Extracts from long bone cultures were prepared according to the method described by Eeckhout et al (31); 0.1 ml CNTN buffer (10mM Cacodylate/HCl (pH 6.0), 1 M NaCl/Triton X-100 (0.1 mg/ml), 1 μM $ZnCl_2/NaN_3$ (0.1 mg/ml)) was added to radii/ulnae, sonificated and agitated by gentle oscillation at 4°C for 24 hr. Next, samples were centrifuged and supernatants were assayed for collagenase activity.

Collagenase assay

Collagenase activity was measured as previously described (32). In brief, latent collagenase was activated using *p*-aminophenylmercuric acetate (APMA, 0.1 mM); enzyme activity was inhibited by ethylenediaminetetraacetic acid (EDTA, 3 mM). 20 μl medium and 180 μl buffer (50 mM Tris, 10 mM $CaCl_2$, 0.1 M NaCl, pH 7.6) in the absence or presence of APMA and/or EDTA was added to 25 μg [^{14}C]-labeled fibrillar collagen (2,500 dpm) and incubated for 24 hr at 35°C. Each sample was assayed in triplicate. Following the incubation period the non-digested collagen was precipitated with 90% ethanol (4°C), and the radioactivity of the supernatant counted using a Packard scintillation counter with 92% efficiency for [^{14}C]. After subtraction of background the data were expressed as units collagenase/well, one unit collagenase being defined as the amount of collagenase degrading 1 μg collagen/min at 35°C.

Immunohistochemistry

Bone biopsies and fixation: A human bone specimen (Os Naviculare) obtained during orthopaedic surgery was fixed for 24 hrs in 4% phosphate-buffered formaldehyde pH 6.9 under vacuum and dehydrated in ascending ethanols for one night prior to a three steps impregnation in methylmethacrylate (MMA) monomer (Merck) for at least 10 hours. Specimen block was impregnated in: 80% (vol/vol) stabilized MMA, 0,4% Perkadox (Akzo Chemicals Netherlands), 20% (vol/vol) Plastoid N (Röhm Pharma Germany) for 2 hours under vacuum and embedded at 37°C overnight. Sections (5 μm) were cut using a Reichert-Jung Polycut S sliding microtome equipped with a Reichert-Jung tungsten carbide knife 40° and mounted on gelatin-chromium-alum coated slides.

Immunohistology: Sections were deacrylated in a 1:1 mixture of xylene and chloroform for 30 minutes and next dipped in xylene, rehydrated, decalcified with 1% acetic acid for 10 minutes, and rinsed two times with distilled water. Endogenous peroxidase activity was inhibited by a 10:1 mixture of methanol and 30% H_2O_2 for 25 minutes, followed by two water washes. Sections were incubated with primary antibody (1:400 - except BMP2 H3B2 1:20) in 0.02 % TPBS (PBS pH 7.4 containing 0.1 % Triton X-100 and 1.5 % BSA) for 1 hour and washed for 5 minutes with three changes of 0,1% TPBS. Primary antibody was detected using a Biogenex Stravigen Multilink HRP kit (San Ramon, CA, USA). Sections were counterstained with a standard hematoxilin-eosin (HE) method. Control sections were stained, by replacing primary antibody by PBS and showed no background staining.

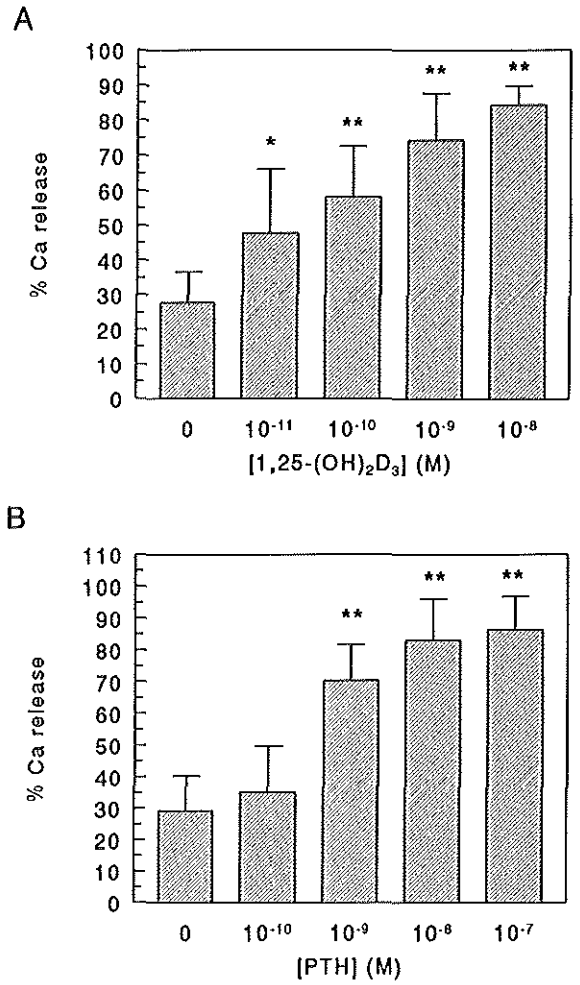

Figure 1

The effects of the systemic factors 1,25-$(OH)_2D_3$ and PTH on bone resorption in fetal mouse long bones. Prelabelled fetal mouse radii/ulnae were incubated with the indicated concentrations A) 1,25-$(OH)_2D_3$ and B) PTH for 6 days and the release of ^{45}Ca into the medium was determined. % ^{45}Ca release is a measure for bone resorption and was assessed as described in Materials and Methods. Data are presented as mean ± SD of 12 cultures. * $p<0.005$, ** $p<0.0001$ versus control.

RESULTS

Effects of local factors on the 1,25-$(OH)_2D_3$ and PTH enhancement of bone resorption

The systemic factors PTH and 1,25-$(OH)_2D_3$ stimulate bone resorption, measured as % ^{45}Ca release into the medium, in a dose dependent way in fetal

mouse radii/ulnae after 3 (data not shown) and 6 days of incubation (Figure 1). The effects were most pronounced after 6 days of incubation. Maximal stimulation of bone resorption was observed at 10^{-8} M-10^{-7} M for 1,25-$(OH)_2D_3$ and PTH.

Incubation of fetal mouse long bones with the locally produced factor TGFß caused a small, but significant inhibition of basal bone resorption after 6 days (Figure 2). Maximal induction of bone resorption by 1,25-$(OH)_2D_3$ (10^{-8} M) was reduced after coincubation with TGFß in a dose-responsive way with 1, 10 and 100 ng/ml TGFß (Figure 2A). However, coincubation with 1000 ng/ml TGFß did not affect the 1,25-$(OH)_2D_3$ enhancement of bone resorption. After 3 days of incubation the effects were less pronounced, but similar, compared to 6 days of incubation (data not shown). TGFß did not affect PTH enhanced bone resorption neither after 3 nor after 6 days (Figure 2B).

BMP-2, which belongs to the TGFß superfamily, had a completely different effect on 1,25-$(OH)_2D_3$-stimulated bone resorption (Figure 3). After 6 days of incubation, BMP-2 did not affect basal bone resorption at concentrations of 1 and 10 ng/ml. However, 100 ng/ml BMP-2 caused a small reduction in basal bone resorption, whereas 1000 ng/ml BMP-2 increased bone resorption to similar levels as 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-8} M PTH. 1,25-$(OH)_2D_3$ stimulation of bone resorption was enhanced in a synergistic manner after coincubation with the concentrations 10 and 100 ng/ml of BMP-2 (Figure 3A), whereas coincubation of 1000 ng/ml BMP-2 and 1,25-$(OH)_2D_3$ led to a similar level of bone resorption as determined after incubation with either BMP-2 or 1,25-$(OH)_2D_3$ alone (Figure 3A). This was also observed after combined treatment of 1000 ng/ml BMP-2 with PTH (Figure 3B). BMP-2 at concentrations effective in combination with 1,25-$(OH)_2D_3$ did not affect the PTH-induced increase in bone resorption (Figure 3B). These effects of BMP-2 were also observed after 3 days of incubation, however less pronounced than after 6 days of incubation (data not shown). IGF-I did not affect basal bone resorption of fetal mouse radii/ulnae after 3 and 6 days. However, in combination with 1,25-$(OH)_2D_3$ IGF-I caused a dose-responsive inhibition of the 1,25-$(OH)_2D_3$ stimulation of bone resorption (Figure 4A). In contrast to TGFß and BMP-2, IGF-I inhibited PTH-stimulated bone resorption (Figure 4B). However, the PTH-stimulated bone resorption is about 10 times less sensitive to IGF-I than the 1,25-$(OH)_2D_3$-stimulated bone resorption (Figure 4). The effects of IGF-I on 1,25-$(OH)_2D_3$- and PTH-stimulated bone resorption were also observed after 3 days of incubation, however, less pronounced than after 6 days (data not shown).

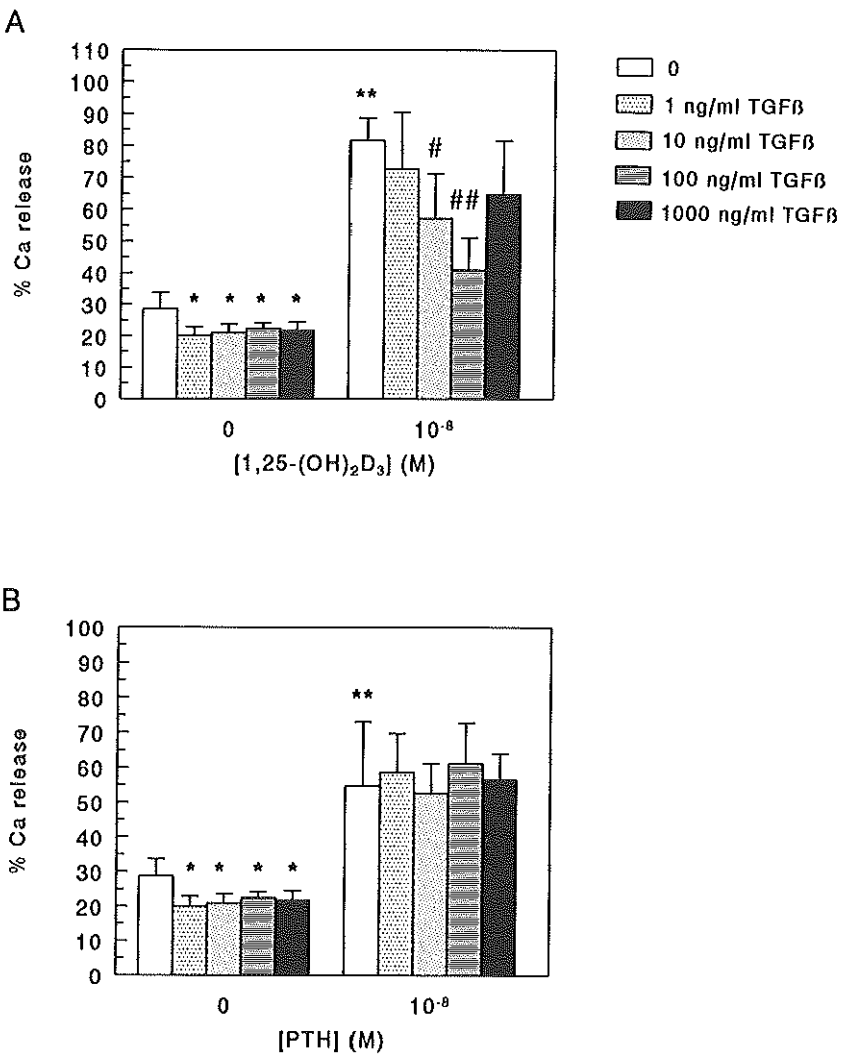

Figure 2

The effects of TGFß on 1,25-$(OH)_2D_3$- and PTH-stimulated bone resorption in fetal mouse long bones. Prelabelled radii/ulnae were incubated with several concentrations of TGFß (0-1000 ng/ml) in the absence or presence of A) 10^{-8} M 1,25-$(OH)_2D_3$, or B) 10^{-8} M PTH for 6 days. % ^{45}Ca release is a measure for bone resorption. Data are presented as mean ± SD of 12 cultures. * $p<0.01$, ** $p<0.0001$ versus control. # $p<0.05$, ## $p<0.0001$ calculated as significance of interaction.

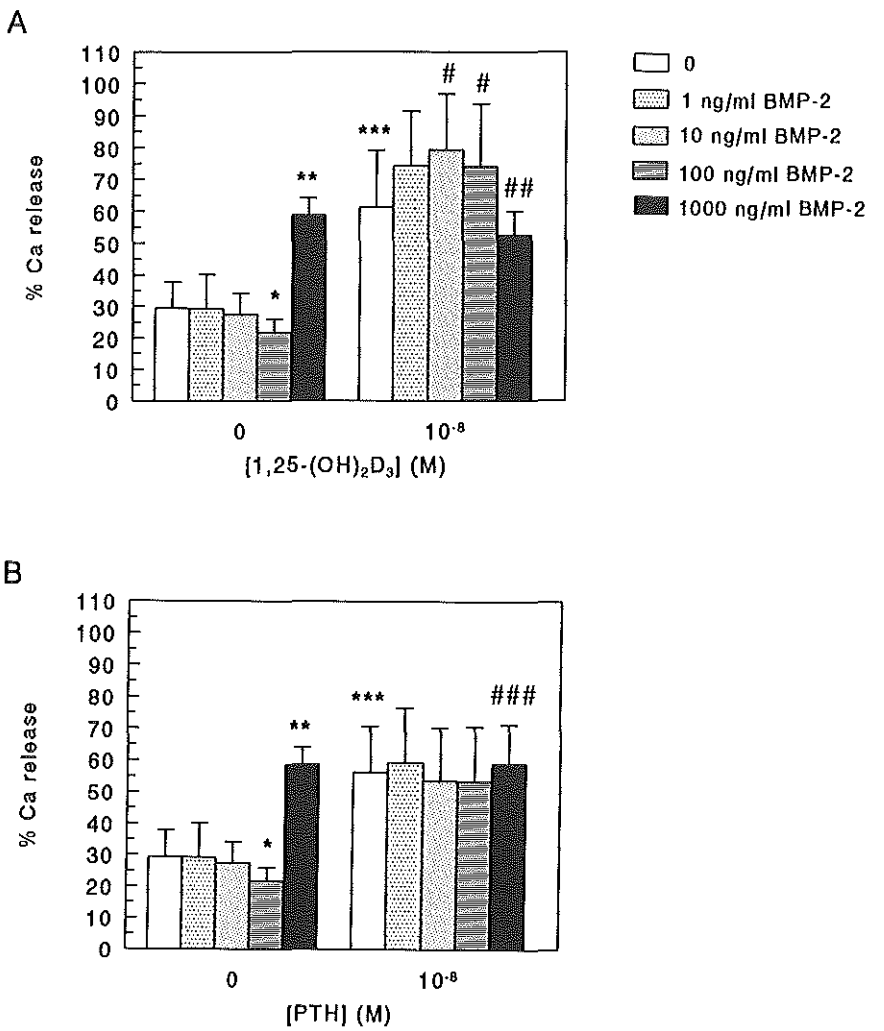

Figure 3

Effects of BMP-2 on 1,25-$(OH)_2D_3$- and PTH-stimulated bone resorption in fetal mouse long bones. Prelabelled radii/ulnae were incubated with several concentrations of BMP-2 (0-1000 ng/ml) in the absence or presence of A) 10^{-8} M 1,25-$(OH)_2D_3$, or B) 10^{-8} M PTH for 6 days. % ^{45}Ca release is a measure for bone resorption. Data are presented as mean ± SD of 12 cultures. * $p<0.05$, ** $p<0.0001$, *** $p<0.005$ versus control. # $p<0.05$ ## $p<0.0001$, ### $p<0.005$ calculated as significance of interaction.

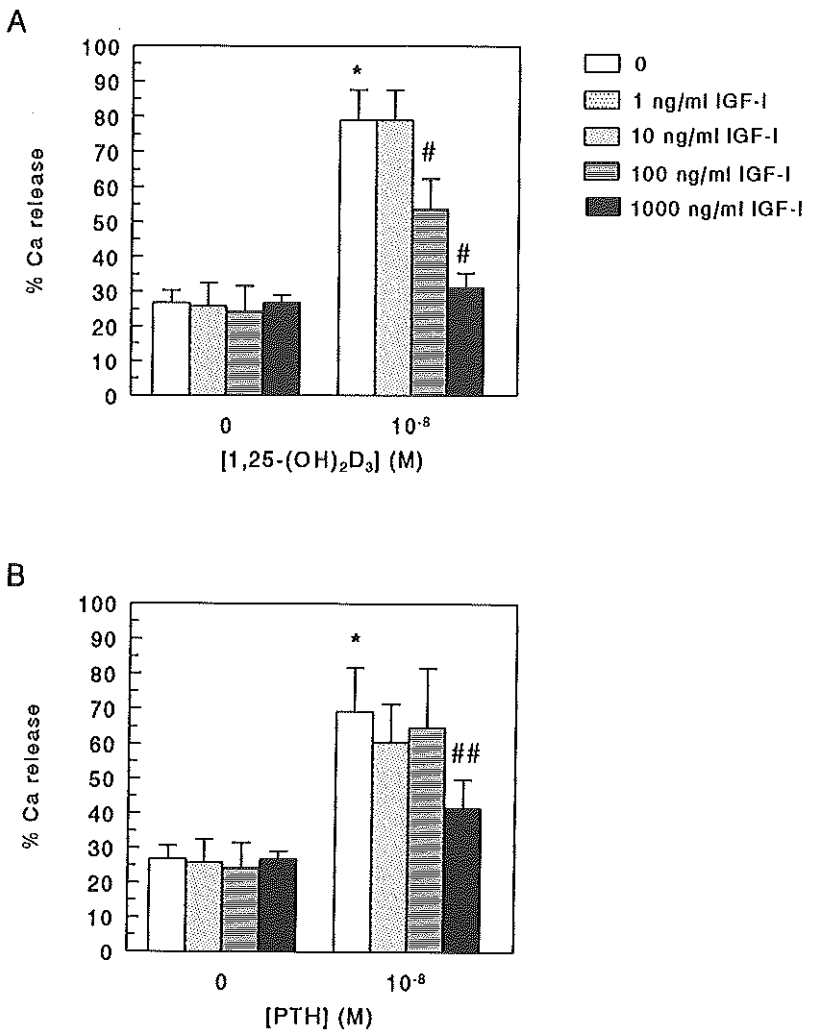

Figure 4

The effects of IGF-I on 1,25-$(OH)_2D_3$- and PTH-stimulated bone resorption in fetal mouse long bones. Prelabelled radii/ulnae were incubated with several concentrations of IGF-I (0-1000 ng/ml) in the absence or presence of A) 10^{-8} M 1,25-$(OH)_2D_3$, or B) 10^{-8} M PTH for 6 days. % ^{45}Ca release is a measure for bone resorption. Data are presented as mean ± SD of 12 cultures. * $p<0.0001$ versus control. # $p<0.0001$, ## $p<0.005$ calculated as significance of interaction.

The effects of locally produced factors and 1,25-$(OH)_2D_3$ on osteocalcin content and release into medium of cultured fetal mouse long bones

Osteocalcin has been suggested to play an important role in bone resorption by enhancing the recruitment and differentiation of osteoclast precursors (33,34). 1,25-$(OH)_2D_3$ is a potent stimulator of osteocalcin production by osteoblasts. Moreover, we have recently demonstrated that TGFß potently inhibits 1,25-$(OH)_2D_3$-stimulated osteocalcin production in both human and rat osteoblastic cells (27,35). Especially 1,25-$(OH)_2D_3$-enhanced bone resorption was affected in the presence of the three growth factors. Therefore, we examined whether a relationship exists between the effects of TGFß, BMP-2 and IGF-I on 1,25-$(OH)_2D_3$-enhanced bone resorption and their effects on osteocalcin production in fetal mouse long bones. Osteocalcin protein levels were measured in the medium and extracts of cultured long bones after 3 and 6 days of incubation with the various growth factors in the absence or presence of 1,25-$(OH)_2D_3$. Surprisingly, 1,25-$(OH)_2D_3$ itself caused a dose-dependent reduction of the osteocalcin level in the medium of fetal mice long bones both after 3 and 6 days of incubation, whereas bone resorption is enhanced by 1,25-$(OH)_2D_3$ (Figure 1). Figure 5 shows the cumulative effect of 1,25-$(OH)_2D_3$ on osteocalcin level in the medium after 6 days. In extracts, using two different extraction methods, the amount of osteocalcin was detectable, but low and no significant change was observed after incubation with 1,25-$(OH)_2D_3$ (data not shown). TGFß alone strongly reduced basal osteocalcin levels in long bone cultures. After coincubation of TGFß and 1,25-$(OH)_2D_3$ osteocalcin levels were reduced to a lesser extent than after incubation with TGFß alone, but were lower than after incubation with 1,25-$(OH)_2D_3$ alone (Figure 6A). IGF-I affected neither basal nor 1,25-$(OH)_2D_3$-reduced osteocalcin levels (Figure 6B). BMP-2 did not affect basal osteocalcin levels in the medium of fetal mouse long bone cultures. However, BMP-2 further enhanced the 1,25-$(OH)_2D_3$-induced reduction in osteocalcin levels (Figure 6C).

The inhibitory effect of 1,25-$(OH)_2D_3$ on osteocalcin levels in medium of fetal mouse long bone cultures is contradictory to the stimulatory effect of 1,25-$(OH)_2D_3$ on osteocalcin production in rat and human osteoblast-like cells. To address the possibility of species specificity, we investigated the effect of 1,25-$(OH)_2D_3$ on osteocalcin production in the neonatal mouse osteoblast-like cell line MC3T3. Similar to the fetal mouse long bone cultures, 1,25-$(OH)_2D_3$ reduced osteocalcin levels in medium as well as in cellular extracts of MC3T3 cells (Figure 7).

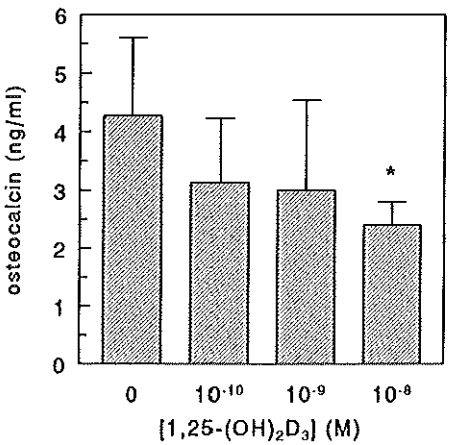

Figure 5

The effect of 1,25-$(OH)_2D_3$ on osteocalcin level of fetal mouse long bones. Fetal mouse radii/ulnae were incubated with several concentrations of 1,25-$(OH)_2D_3$ and osteocalcin levels were measured in the medium after 6 days. Data are presented as mean ± SD of 6 cultures. * $p<0.05$ versus control.

The effects of TGFß and 1,25-$(OH)_2D_3$ on collagenase level in fetal mouse long bones

In addition to the investigation of osteocalcin as a possible mediator of the regulation of bone resorption, we studied the regulation of the activity of collagenase, an enzyme demonstrated to be involved in bone matrix degradation (36) by 1,25-$(OH)_2D_3$ in fetal mouse long bone cultures. 1,25-$(OH)_2D_3$, however, did not affect collagenase activity in these cultures (Table 1). Additionally, we investigated the effects of one of the locally produced growth factors, TGFß, on collagenase activity. Collagenase activity was not affected by TGFß (Table 1). These observations do not support a role of this enzyme in the inhibitory effects of TGFß on basal and 1,25-$(OH)_2D_3$-enhanced bone resorption.

Localization of locally produced factors in normal human bone

As interactions between local factors and systemic factors occur in the regulation of bone resorption, it is of interest to determine the presence and localization of these locally produced factors in normal bone. The purpose of this examination was not to determine their precise localization in relation to known bone histological and histomorphometric parameters, but to assess whether these factors

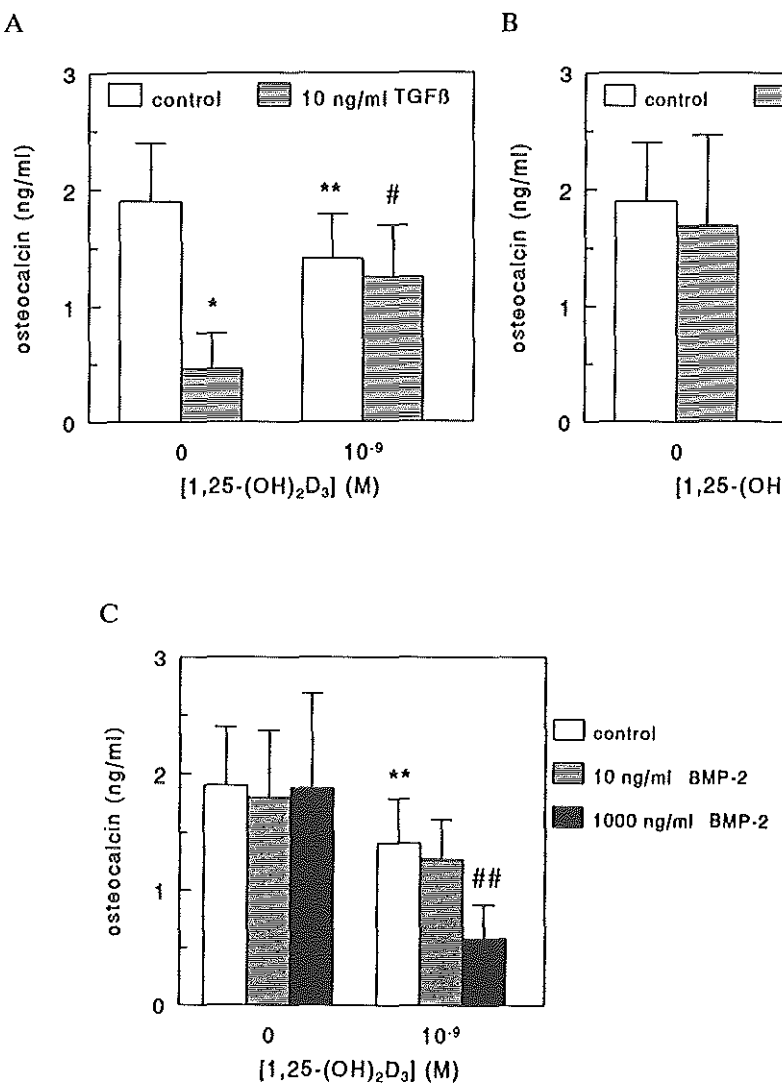

Figure 6

The effects of TGFß, BMP-2 and IGF-I in the absence or presence of 1,25-$(OH)_2D_3$ on osteocalcin level in the medium of fetal mouse long bones. Fetal mouse long bones were incubated with A) 10 ng/ml TGFß, B) 100 ng/ml IGF-I, and C) 10 or 1000 ng/ml BMP-2 in the absence or presence of 10^{-8} M 1,25-$(OH)_2D_3$ for 6 days. Osteocalcin level was measured after 6 days. Data are presented as mean ± SD of 7 cultures. * $p<0.0001$, ** $p<0.05$ versus control. # $p<0.001$, ## $p<0.05$ significance of interaction.

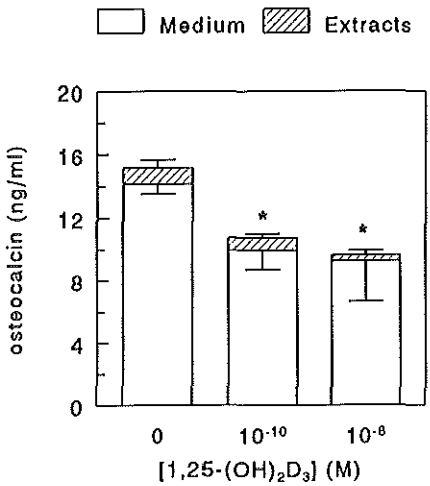

Figure 7

The effect of 1,25-$(OH)_2D_3$ on osteocalcin level in the mouse osteoblast-like cell line MC3T3. MC3T3 cells were cultured for 2 weeks till confluency followed by the incubation with 10^{-10} M or 10^{-8} M 1,25-$(OH)_2D_3$ for 48 hr. Osteocalcin levels were measured in medium as well as cell extracts of MC3T3 cells. Data are presented as mean ± SD of 2 cultures. * $p<0.05$ versus control.

TABLE 1

The effects of TGFß and 1,25-$(OH)_2D_3$ on collagenase activity in fetal mouse long bones after 6 days.

	collagenase activity (Units)
control	6.98 ± 0.84
1,25-$(OH)_2D_3$	7.07 ± 1.30
TGFß	7.07 ± 3.07
1,25-$(OH)_2D_3$ + TGFß	7.07 ± 2.8

Fetal mouse radii/ulnae were incubated with 10 ng/ml TGFß and 10^{-8} M 1,25-$(OH)_2D_3$ for 6 days. A medium change was performed after 3 days. Collagenase was determined in radii/ulnae extracts after 6 days of incubation.

are diffusely present throughout bone or whether their presence is restricted to particular sites. We investigated the localization of TGFß type 2, TGFß type 3, BMP-2 and IGF-I in human adult bone by immunohistochemistry. As shown in Figure 8, all factors are highly expressed in particular areas and are not diffusely present throughout bone.

DISCUSSION

The data obtained in the present study clearly support the hypothesis that growth factors present in the bone matrix regulate bone resorption stimulated by 1,25-$(OH)_2D_3$ and PTH. The 1,25-$(OH)_2D_3$- and PTH-enhanced bone resorption is regulated in a growth factor specific manner. Moreover, the effects of TGFß, BMP-2, and IGF-I are hormone specific. We observed that TGFß inhibits 1,25-$(OH)_2D_3$- but not PTH-stimulated bone resorption in fetal mouse long bones. BMP-2 stimulated 1,25-$(OH)_2D_3$-induced bone resorption whereas the effect of PTH on bone resorption was not affected. IGF-I inhibited both 1,25-$(OH)_2D_3$- and PTH-enhanced bone resorption although with different sensitivities. These data implicate that growth factors are involved in the control of the extent of bone resorption in response to 1,25-$(OH)_2D_3$ and/or PTH. To gain insight into the localization of these growth factors in the bone matrix, the presence and distribution of TGFß type 2, TGFß type 3, BMP-2 and IGF-I in adult human bone were examined. Immunohistochemical analysis revealed that the presence of these growth factors is restricted to specific sites and that they are not diffusely present throughout the bone. Thus, the amount and type of growth factor present in the bone matrix may eventually cause less or more profound resorption in response to 1,25-$(OH)_2D_3$ and/or PTH at particular sites. For example, 1,25-$(OH)_2D_3$-induced bone resorption at a TGFß-positive site may be limited whereas at a TGFß-negative site resorption can go on. The proposed model requires that growth factors should be released from the bone matrix during resorption and must still be biologically active or in a configuration that can be activated. This seems not unlikely because TGFß is stored in a latent form in the bone matrix (37,38) and IGF-I binds to IGF-BPs which are capable of binding the extracellular matrix (21,39,40). These features can have protective effects for the growth factor which may result in the eventual release of active growth factors. This is supported by the *in vitro* observation that several factors that stimulate bone resorption at the same time enhance TGFß activity in the culture medium (41). This

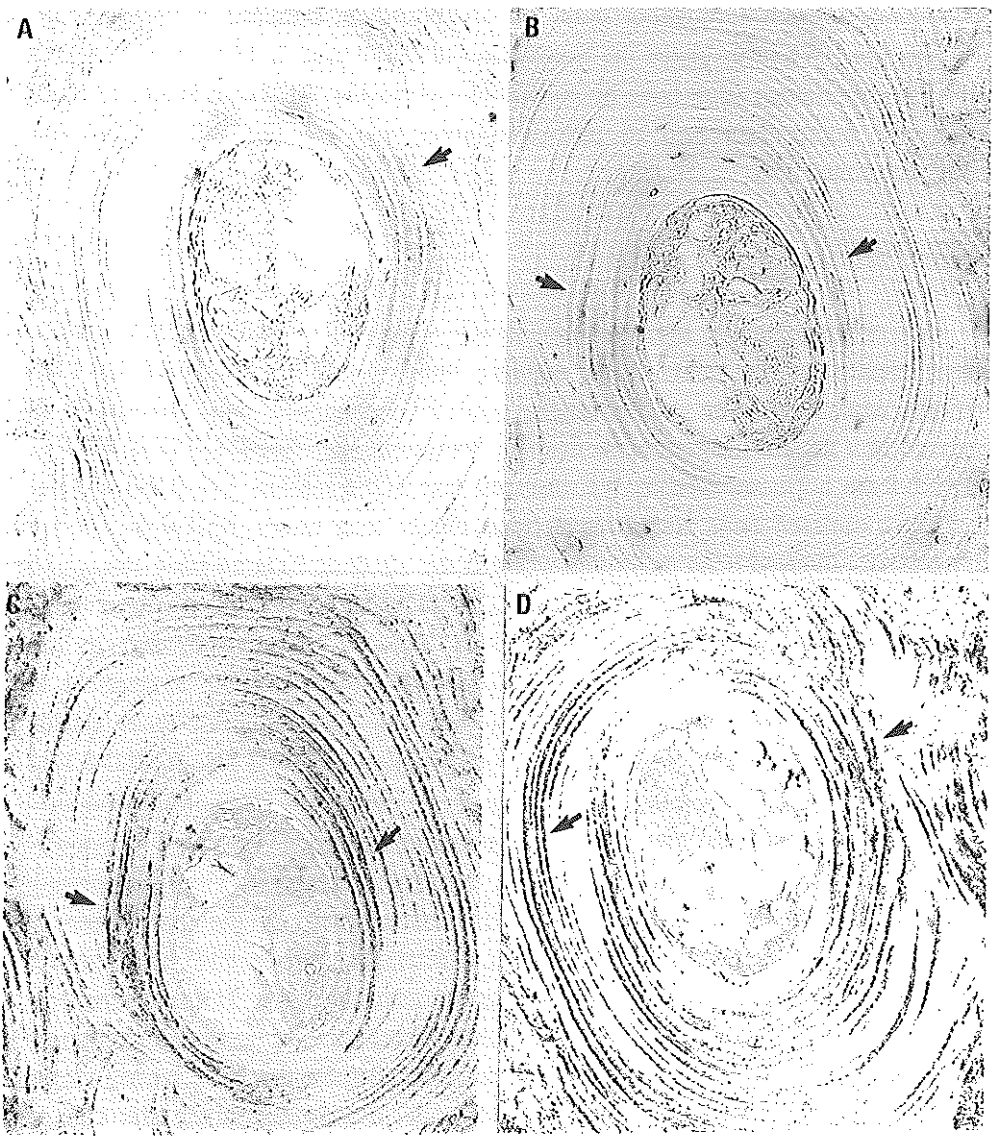

Figure 8

Presence and distribution of TGFß type 2, TGFß type 3, BMP-2 and IGF-I in normal human adult bone. Immunohistochemical analysis was performed as described in Materials and Methods, using antibodies specific for A) TGFß type 2, B) TGFß type 3, C) BMP-2 and D) IGF-I. Arrows indicate the presence of the various growth factors.

indeed suggests that TGFß, but possibly also the other growth factors, may serve as a local feedback regulatory mechanism for bone resorption.

The present results about differential regulation of 1,25-$(OH)_2D_3$ and PTH stimulation of bone resorption by the growth factors tested point to different bone resorptive mechanisms for 1,25-$(OH)_2D_3$ and PTH. This supports the observation that 1,25-$(OH)_2D_3$, unlike PTH, affects precursors of the osteoclast lineage (42-44). Our data that TGFß inibits 1,25-$(OH)_2D_3$ but not PTH stimulation of bone resorption seems to be in line with these observations because TGFß is a potent inhibitor of 1,25-$(OH)_2D_3$-induced osteoclast-like cell formation in long-term human bone marrow cultures, both by inhibiting proliferation and differentiation of early precursors as well as by inhibiting fusion of mononuclear cells to form osteoclasts (45). In addition, in fetal rat long bones TGFß inhibition of 1,25-$(OH)_2D_3$-stimulated bone resorption was suggested to occur via inhibition of osteoclast precursor proliferation (46).

To gain more insight into the mechanisms whereby TGFß, BMP-2 and IGF-I affect 1,25-$(OH)_2D_3$-stimulated bone resorption we investigated the effects on osteocalcin production and collagenase activity in fetal mouse long bone cultures. Previously, osteocalcin has been suggested to play a stimulatory role in bone resorption by enhancing the recruitment and/or differentiation of osteoclasts (33,34). This is supported by evidence that osteocalcin deficiency leads to an increase in the amount of bone matrix deposition resulting in a 50% increased cortical thickness, replacement of the growth plate and epiphysal cartilage by newly formed bone and a significant increase in bending strength of long bones (47). In rat and human osteoblast-like cells osteocalcin production is strongly stimulated by 1,25-$(OH)_2D_3$, and this stimulation has been shown to be inhibited by TGFß (26,27,35,48). This observation in rat osteoblast-like cells parallels the regulation of fetal rat long bone resorption by 1,25-$(OH)_2D_3$ and TGFß. However, in fetal mouse long bones we observed an inhibitory effect of 1,25-$(OH)_2D_3$ on osteocalcin content, in contrast to the stimulatory effect of 1,25-$(OH)_2D_3$ on fetal mouse bone resorption. TGFß alone strongly reduced basal osteocalcin level and in combination with 1,25-$(OH)_2D_3$ osteocalcin level was reduced to a lesser extent than after incubation with TGFß alone. To investigate whether the difference between the effects of 1,25-$(OH)_2D_3$ in rat/human osteoblast-like cells and fetal mouse long bones was due to a species difference, a difference between isolated osteoblasts and long bone culture or a difference between fetal and non-fetal regulation, we also studied the effect of 1,25-

$(OH)_2D_3$ on osteocalcin production in the neonatal mouse osteoblast-like cell line MC3T3. Like in fetal mouse long bone cultures, in MC3T3 cells osteocalcin production was reduced in the presence of 1,25-$(OH)_2D_3$. However, Gundberg et al. showed in an *in vivo* study that administration of 1,25-$(OH)_2D_3$ to mice increased serum osteocalcin level (49). Combining this observation with our data suggests that this is due to release from bone matrix during resorption rather than new synthesis in response to 1,25-$(OH)_2D_3$. The difference between rat/human and mouse suggests species specificity in the regulation of osteocalcin production by 1,25-$(OH)_2D_3$. This is supported by data of Sztajnkrycer et al. (50), who reported that in ROS 17/2.8 cells stably transfected with the mouse osteocalcin genes, 1,25-$(OH)_2D_3$ also reduced mouse osteocalcin production, whereas the endogenous rat osteocalcin gene was stimulated by 1,25-$(OH)_2D_3$. Kesterson et al. (51) introduced the human osteocalcin gene promoter fused to the chloramphenicol acetyltransferase gene into the germ line of mice and showed reduction of CAT activity after a low vitamin D_3 diet, while 1,25-$(OH)_2D_3$ supplementation restored and enhanced CAT activity over control values. These data show that the differences observed between rat/human and mouse are not due to differences in the availability of factors important for the regulation of the osteocalcin gene. Remarkable is the difference in the 5' half site of the vitamin D responsive elements (VDREs) between the rat (GGGTGAnnnAGGACA; 52-54), human (GGGTGAnnnGGGGCA; 55,56) and mouse (GGGCAAnnnAGGACA; 57) VDREs of the osteocalcin genes. Underlined are the nucleotides in the mouse VDRE that differ from both the rat and human genes. This dinucleotide difference in the VDRE may lead to a change in its ability to bind nuclear proteins and thus, eventually in a change in regulation of transcription. Irrespective of the lack of knowledge on the precise mechanism, our data do not support a major role for osteocalcin in the 1,25-$(OH)_2D_3$ regulation of bone resorption in fetal mouse long bones.

Another candidate protein that could be the mediator in the inhibitory effect of TGFß on 1,25-$(OH)_2D_3$ enhancement of bone resorption is collagenase (58,59). Collagenase has been shown to be an important mediator in calvarial bone resorption (60). In osteoblast-like cells collagenase activity is stimulated by 1,25-$(OH)_2D_3$ and PTH (61-64). In mouse calvarial osteoblast cultures TGFß has been shown to inhibit 1,25-$(OH)_2D_3$ stimulated collagenolysis (65). However, in our study in fetal mouse long bones, we did not observe changes in collagenase activity after incubation with 1,25-$(OH)_2D_3$ for 6 days. Collagenase does not seem to play a role in the 1,25-

$(OH)_2D_3$ enhancement of resorption. The discrepancy between our results and previous reports may be caused by differences in the regulation of collagenase activity between calvaria and long bones. Furthermore, a time-related aspect may be important. The regulation of the production of tissue inhibitor of metalloproteinase-I, an enzyme reducing the activity of collagenase, may provide a feedback mechanism, whereby after 6 days 1,25-$(OH)_2D_3$- or TGFß-induced changes are counteracted.

In conclusion, this study shows growth factor specificity in the regulation of 1,25-$(OH)_2D_3$ and PTH enhanced bone resorption as well as hormone specificity, as shown by the differential effects of each growth factor on 1,25-$(OH)_2D_3$ and PTH stimulation of bone resorption. Furthermore, we show that the expression of TGFß type 2, TGFß type 3, BMP-2 and IGF-I is restricted to specific sites in bone. Together with the bone resorption results, this suggests that locally produced factors define the action of systemic factors and are thereby important in the regulation of the architecture of bone. However, the mechanism by which 1,25-$(OH)_2D_3$- and PTH-enhanced bone resorption is regulated by TGFß, BMP-2 and IGF-I remains unclear. The present data on osteocalcin and collagenase activity do not support a major role for these proteins in the regulation of bone resorption at least in the model system used in this study.

AKNOWLEDGMENTS

We thank Dr. C.W.G.M. Löwik for stimulating discussions and incubations of MC3T3 cells. Furthermore, we thank The Genetics Institute Cambridge, MA, USA for providing us with bone morphogenetic protein-2 and the mouse monoclonal antibody h3b2/17.8.1 against bone morphogenetic protein-2 (BMP-2) and Prof. R. Bouillon, Leuven, Belgium, for mouse osteocalcin antiserum and the protocol for the mouse osteocalcin assay.

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CHAPTER 7

DISTINCT CONFORMATIONS OF VDR/RXRα HETERODIMERS ARE SPECIFIED BY DINUCLEOTIDE DIFFERENCES IN THE VITAMIN D RESPONSIVE ELEMENTS OF THE OSTEOCALCIN AND OSTEOPONTIN GENES

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Submitted for publication

ABSTRACT

The 1α,25-dihydroxyvitamin D_3 (VD3)-dependent stimulation of osteocalcin (OC) and osteopontin (OP) gene transcription in bone tissue is mediated by interactions of trans-activating factors with distinct VD3 responsive elements (VDREs). Sequence variation between the OC- and OP-VDRE steroid hormone half-elements provides the potential for recognition by distinct hormone receptor homo- and heterodimers. However, the exact composition of endogenous VD3 induced complexes recognizing the OC- and OP-VDREs in osteoblasts has not been definitively established. To determine the identity of these complexes, we performed gel shift immuno-assays with nuclear proteins from ROS 17/2.8 osteoblastic cells using a panel of monoclonal antibodies. We show that VD3 inducible complexes interacting with the OC- and OP-VDREs represent two distinct heterodimeric complexes, each composed of the Vitamin D Receptor (VDR) and the Retinoid X Receptor α (RXR). While the OC- and OP-VDR/RXRα heterodimers are immunoreactive with both RXRα and VDR antibodies (i.e., 9a7γ and VD2F12), only the OC-VDRE complex is recognized by a specific monoclonal antibody (IVG8C11) which contacts an epitope in the VDR DNA binding domain (amino acids 57-164). By systematically introducing a series of point-mutations in the OC-VDRE, we find that two internal nucleotides of the proximal OC-VDRE half-site (nt -449 and -448 5'-AGGACA) determine differences in VDR immunoreactivity. This result provides a novel line of evidence for polarity of VDR/RXRα binding to OC- and OP-VDREs, with the proximal half-elements being recognized by the VDR, and further suggests that the DNA binding domain of the VDR adopts different protein conformations when contacting distinct VDREs. Distinctions between the OC- and OP-VDR/RXRα complexes may reflect specialized requirements for VD3 regulation of OC and OP gene expression in response to physiological cues mediating osteoblast differentiation.

INTRODUCTION

1α,25-dihydroxyvitamin D_3 (VD3) is essential for the maintenance of calcium homeostasis and skeletal development and exerts most of its effects through the vitamin D receptor (VDR)(1). VDR containing transcription factor complexes trans-activate osteoblast related genes via VD3 responsive cis-acting elements (VDREs). Transcriptional regulation of the VD3 responsive osteocalcin (OC) and osteopontin (OP) genes, which encode major non-collagenous proteins in bone, functionally contributes to

bone formation and resorption. The well-characterized VDREs located in the promoters of the OC and OP genes represent important paradigms for understanding VD3 mediated transcriptional mechanisms in bone tissue.

The VDR belongs to the superfamily of nuclear steroid hormone receptors (1-4) which also includes retinoic acid receptors (RAR and RXR), thyroid hormone receptors (TR) and several orphan receptors. The VDR is capable of forming homodimers, as well as heterodimers with RXR α and RXR β (5-10), that bind to direct repeats of hexameric half elements (e.g., 5'-PuG(G/T)TCA) with distinct spacing requirements (11-15). In addition, in vitro binding site selection experiments have revealed that VDR/VDR homodimers and VDR/RXR heterodimers have different nucleotide preferences within the VDRE hexamers (14,15). Moreover, distinct VDREs, which have the potential to interact with different receptor homo- and hetero-dimers, have been identified in the promoters of several genes; including the human (16,17) and rat osteocalcin (18-21), mouse osteopontin (22), rat 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 24-hydroxylase (23-26), as well as the mouse calbindin D28k (27) and chicken β3 integrin (28) genes.

Several studies have established the potential for functional interactions between distinct VDR and/or RXR complexes at the OC- and OP-VDREs. For example, heterodimerization of the VDR with RXR-related auxiliary nuclear factors is required

Table 1

rat OC-VDRE	5'-GGGTGA ATG AGGACA-3'
mouse OP-VDRE	5'-GGTTCA CGA GGTTCA-3'
human OC-VDRE	5'-GGGTGA ACG GGGGCA-3'
rat calbindin D-28K VDRE	5'-GGGGGA TGT GAGGAG-3'
rat cytochrome $P450_{24}$ VDRE	5'-AGGTGA GTG AGGGCG-3'
avian integrin $ß_3$ VDRE	5'-GAGGCA GAA GGGAGA-3'
rat calbindin D-9K	5'-GGGTGT CGG AAGCCC-3'

The table shows nucleotide sequences of the hexameric steroid hormone half elements (underlined) in the OC and OP VDREs, as well as VDREs identified in other genes as indicated (see text for references).

for binding to the OC-VDRE (29-40). However, many of the structural parameters of receptor interactions with the OC- and OP-VDREs have been investigated by in vitro binding studies using VDR and selected RXR proteins, which were obtained by bacterial expression, in vitro transcription/translation with crude cell lysates, or by virally mediated expression in insect cells; several studies also employed lysates derived from non-osseous cells in which candidate receptor proteins were over-expressed, or used synthetic oligonucleotides that do not resemble native VDREs. Also, functional aspects of VDR/RXR interactions have been analyzed by transient transfection experiments in non-osseous cells using over-expression plasmids encoding VDR or RXR, and artificial chimeric reporter gene constructs. These excellent reductionist approaches have provided key insights into the potential mechanisms by which VDR mediates transcriptional enhancement of responsive genes. However, these approaches do not provide physiologically relevant information on the VDR containing complexes present in osseous cells, which is required to gain insight into the regulation and regulatory roles of the VDR in bone tissue. Therefore, the precise compositions of endogenous VD3 containing complexes that interact with the OC- and OP-VDREs and are physiologically responsive to VD3 in osteoblasts, remain to be established.

In this study, using a panel of antibodies against the VDR and RXRs and a systematic series of point mutations within the OC VDRE, we performed a molecular dissection of the interactions of endogenous VDR/RXR heterodimers from osseous cells with VDREs. The results show that the endogenous VDR complexes interacting with OC and OP VDREs represent VDR/RXRα heterodimers. We present a novel immunological line of evidence for the concept that VDREs specify receptor polarity. The VDR recognizes exclusively the proximal steroid half element in both the OC and OP VDREs. Furthermore, we find that immunologically distinct VDR/RXR complexes bind to the OC and OP VDREs, with the conformation of the VDR moiety in each complex depending on specific dinucleotides within the proximal steroid half element. Our findings are consistent with the concept that VDREs dictate receptor dimer specificity and are capable of conferring distinct conformations to DNA bound VDR/RXRα complexes.

MATERIALS AND METHODS

Cell culture

ROS 17/2.8 osteosarcoma cells, kindly provided by Dr. G. Rodan and Dr. S. Rodan (Merck Sharp & Dohme Research Laboratories, West Point, PA) were maintained in Hams F12 medium supplemented

with 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin and 5% fetal calf serum (FCS) at 37°C under 95% air/5% CO_2. For the experiments, cells were seeded in 100 x 20 mm plastic dishes at 0.7 x 10^6 cells/plate in Hams F12 medium containing 5% FCS and cultured for 24 hr. Subsequently, medium was replaced by Hams F12 with 2% charcoal-treated FCS and cells were cultured overnight and then incubated for 24 hr with VD3, TGFβ$_1$ and/or TGFβ$_2$ as indicated in the figure legend. 1,25-$(OH)_2D_3$ was kindly provided by Dr. M. Uskokovic Hoffman-La Roche Inc. TGFß$_1$ was purchased from R&D Systems, Minneapolis, MN. TGFβ$_2$ was kindly provided by Dr. J. Feyen, Sandoz Pharma LtD, Basel, Switzerland.

Immunological reagents

The following VDR antibodies were used in our study: IVG8C11, VD2F12 and 9a7γ. The mouse monoclonal antibodies IVG8C11 and VD2F12 were directed against the porcine VDR, and recognize both human and rat VDR. The epitopes of these antibodies are distinct, and have been mapped to amino acids 57 to 164 spanning the highly conserved DNA binding domain (41). The 9a7γ antibody represents a monoclonal antibody recognizing amino acids 89 to 105.

Several different mouse monoclonal RXR antibodies (42) were used in our experiments. Antibody 4RX-1D12 (in this study referred to as antibody RXRα,β,γ) recognizes the D/E region; this antibody cross-reacts with all three RXR members, RXRα, RXRβ and RXRγ. Antibodies 4RX-3A2 and 83-106-PC13 are specific for RXRα and RXRβ, respectively.

Gel mobility shift assays

The following VDRE-containing oligonucleotides were used in gel mobility shift assays: rat osteocalcin VDRE oligo (19), 5'-CTGCACTGGGTGAATGAGGACATTACTGA-3', and mouse osteopontin VDRE oligo (21), 5'-ACAAGGTTCACGAGGTTCACGTCT-3'; the underlined nucleotides represent the steroid hormone half-sites that mediate binding of nuclear receptor complexes and are shared among distinct VDREs (Table 1). A series of mutant OC-VDRE oligonucleotides (Table 2) was used to investigate which nucleotides in the OC- and OP-VDRE half sites are responsible for differences in immunoreactivity with the IVG8C11 antibody.

Gel-purified oligonucleotides were labeled with [^{32}P] ATP. Nuclear extracts were prepared as described previously (43-45). In brief, cells were scraped in PBS and after centrifugation the cell pellet was reconstituted in a hypotonic lysis buffer (10 mM HEPES, pH7.5, 10mM KCl and 0.2 mM EDTA) supplemented with 0.75 mM spermidine, 0.15 mM spermine, 1 mM EGTA, 1 mM DTT and a broad spectrum of protease inhibitors: 0.2 mM PMSF, 70 μg/ml TPCK, 10 μg/ml trypsin inhibitor, 0.5 μg/ml leupeptin and 1.0 μg/ml pepstatin. 0.5% Nonidet P40 was added and cells were lysed using a dounce homogenizer. After centrifugation nuclear extracts were obtained by incubating nuclei in a hypertonic nuclear extraction buffer (20mM HEPES, pH 7.5, 420 mM KCl, 25% glycerol and 0.2mM EDTA) supplemented with the same reagents previously mentioned for the hypotonic lysis buffer. Desalting was performed by dilution with KN0 buffer (20% glycerol, 0.2 mM EDTA, 0.01% Nonidet-P40, Hepes, pH 7.5).

Protein/DNA binding reactions were performed in a final concentration of 50 mM KCl and 0.5 fmol/μl DNA, 0.1 μg/μl poly(dI-dC)*(dI-dC) and 4 mM DTT. The results of gel shift immuno-assays presented in the figures throughout this study were performed by incubating nuclear protein preparations from VD3 treated cells with VDR antibodies 15 minutes prior to addition of radiolabelled VDRE

Table 2

rOC VDRE	5'-CTGCACTGGGTGAATGAGGACATTACTGA-3'
mOP VDRE	5'-ACAAGGTTCACGAGGTTCACGTCT-3'
OP/OP	5'-CTGCACTGGTTCAATGGGTTCATTACTGA-3'
OC/OP	5'-CTGCACTGGGTGAATGGGTTCATTACTGA-3'
OP/OC	5'-CTGCACTGGTTCAATGAGGACATTACTGA-3'
pm 1G3T	5'-CTGCACTGGGTGAATGGGTACATTACTGA-3'
pm 1G4T	5'-CTGCACTGGGTGAATGGGGTCATTACTGA-3'
pm 3T4T	5'-CTGCACTGGGTGAATGGGTTCATTACTGA-3'
pm 3T	5'-CTGCACTGGGTGAATGAGTACATTACTGA-3'
pm 4T	5'-CTGCACTGGGTGAATGAGGTCATTACTGA-3'

The table shows nucleotide sequences (upper strand shown) of the wildtype OC- and OP-VDREs (the hexameric steroid hormone half elements are underlined), as well as mutant oligonucleotides which were used for determining which nucleotides are responsible for differential immunoreactivity of the OC- and OP-VDRE complexes with the 1VG8C11 VDR antibody. Substitution mutations were designed within the context of OC-VDRE oligonucleotide and the corresponding mutants represent hybrids between the OC- and OP-VDREs. In mutant OP/OP, both the distal and proximal hexamers of the OC VDRE have been substituted for the analogous elements of the OP-VDRE, whereas mutants OC/OP and OP/OC contain substitutions of the proximal or distal hexamer, respectively. The remaining oligonucleotides represent point mutations within the proximal hexamer of the OC-VDRE.

oligonucleotides. We did not observe any differences in the outcome of the experiments when we performed pre- and post-incubation experiments in which the VDR antibody 1VG8C11 was incubated with protein either prior to or after addition of radio-labelled OC- and OP-VDREs (data not shown). Protein/DNA complexes were separated in a 4% polyacrylamide gel (acrylamide : bisacrylamide = 80 : 1) run in 0.5x TBE. Gels were dried and exposed to x-ray film.

RESULTS

Immunologically distinct VDR/RXR complexes interact with OC- and OP-VDREs

The OC and OP vitamin D responsive elements each mediate formation of one main endogenous complex using nuclear proteins from vitamin D treated ROS 17/2.8 rat osteosarcoma cells in gel shift assays (Fig. 1). Competition analysis demonstrates that the OC-VDRE complex is competed by a 100-fold molar excess of both the unlabelled OC-VDRE and OP-VDRE oligonucleotides. Similarly, cross-competition is also observed for the OP-VDRE complex. Thus, the OC- and OP-VDRE complexes detected with

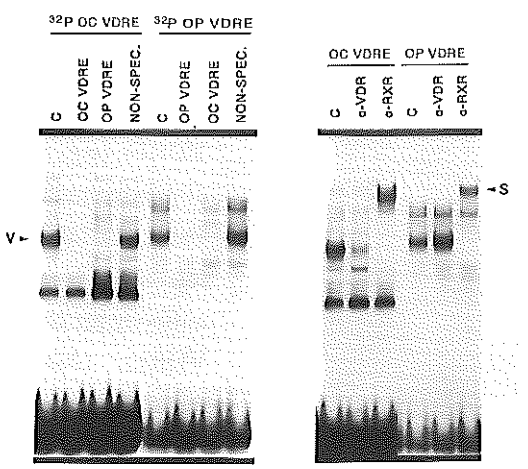

Figure 1

Characterization of VD3 inducible complexes at the OC-and OP-VDREs. The OC- or OP-VDRE probes (as indicated above the lanes) each mediate formation of one main VD3 inducible complex (designated V) when incubated with nuclear protein (approximately 5 μg) from VD3 treated (10^{-8} M, 24 hr) ROS 17/2.8 cells. The left panel represents an oligonucleotide competition assay which was performed using a 100-fold molar excess of unlabelled OC- or OP-VDRE or a non-specific competitor (SP-1). Cross-competition is observed between the OC- and OP-VDRE complexes, indicating similar recognition specificities for DNA binding. The right panel represents a gel shift immuno-assay with nuclear proteins from VD3 treated ROS 17/2.8 cells in the presence of a monoclonal antibody to the VDR (IVG8C11, αVDR) and a polyclonal antibody to RXR (recognizing the α, β, γ subtypes of RXR, α-RXR). Nuclear proteins and antibodies were co-incubated for 15 minutes and subsequently either labelled OC- or OP-VDRE was added to the reaction mixtures. Immunoreactivity of the OC-VDRE complex is indicated by loss of binding with the VDR antibody (IVG8C11), and a supershift (S) with the RXR antibody.

endogenous receptor proteins and accessory factors in osteoblastic cells are mediated by DNA binding activities with similar recognition specificities.

Immunoreactivity of the OC- or OP-VDRE complexes was tested using a specific monoclonal VDR antibody (IVG8C11) and a monoclonal RXR antibody (4RX-1D12), which cross-reacts with the three RXR subtypes RXRα, RXRβ and RXRγ. The VDR antibody inhibits formation of the OC-VDRE complex, whereas the RXR antibody causes a supershift (Fig. 1). In contrast, the OP-VDRE complex is supershifted by the RXR antibody, but formation of this complex is not reduced by the VDR antibody. We also analyzed binding to other functional VDREs (e.g., human OC and rat 1,25-dihydroxyvitamin D_3 24-hydroxylase; see Table 1); the results confirmed the selective recognition of the VDR antibody for complexes bound at distinct VDREs (data not shown). Taken together, these gel shift immuno-assays reveal molecular distinctions between the OC- and OP-VDRE complexes.

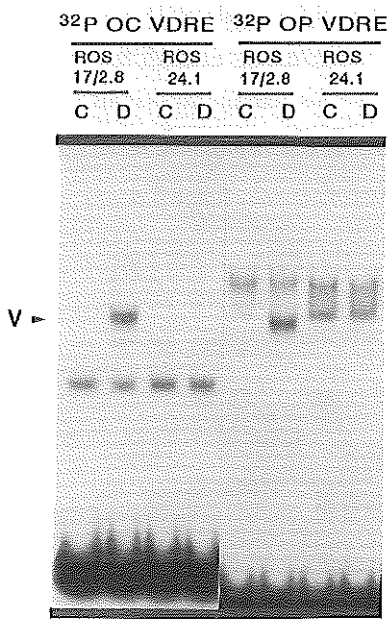

Figure 2

The VD3-responsive complexes of both the OC- and OP-VDREs require the presence of the VDR. As indicated above the lanes, binding reactions were performed with either labelled OC- or OP-VDRE using nuclear extracts that were prepared from either ROS 17/2.8 or ROS 24/1 cells cells after 24 hr incubation with (D) or without (C) 10^{-8} M vitamin D. The VD3-responsive protein/DNA complex observed with nuclear proteins from ROS 17/2.8 cells (designated V) is absent in nuclear extracts from VD3-treated ROS 24.1 cells which lack a functional VDR.

To analyze whether the OP-VDRE complex depends on the presence of VDR, we compared VDRE binding of nuclear proteins from ROS 17/2.8 cells with those from ROS 24/1 cells, which do not contain functional VDR. Nuclear proteins isolated from VD3 treated ROS 24/1 cells do not mediate inducible complexes with the OC- and OP-VDREs (Fig. 2). This result indicates that presence of the VDR is

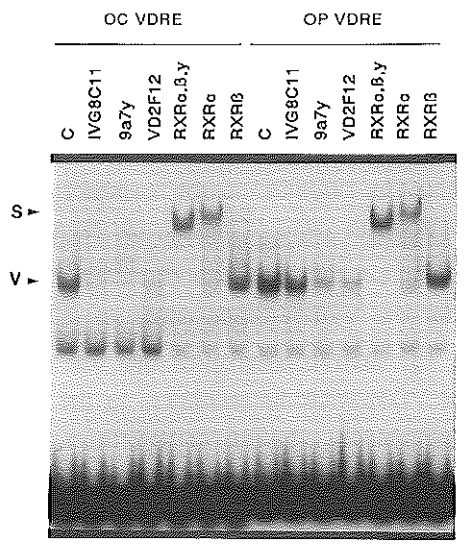

Figure 3

OC- and OP-VDRE complexes display selective immunoreactivity for several VDR antibodies (IVG8C11, 9a7γ and VD2F12) and RXR antibodies recognizing all three RXR subtypes (RXRα, β, γ) or directed against specific RXR subtypes (RXRα or RXRβ). Gel shift assays were performed by pre-incubating nuclear proteins from VD3 treated ROS 17/2.8 cells with the antibodies indicated above the lanes. The VD3-responsive protein/DNA complexes for the OC- and OP-VDREs (V) and the corresponding supershift complexes (S) are indicated by arrowheads. Differential immunoreactivity is evidenced by complete inhibition of the OC-VDRE complex, but not the OP-VDRE complex, with the IVG8C11 antibody. The other two VDR antibodies display more subtle differences in immunoreactivity (i.e., complete versus partial inhibition of binding), whereas the RXR antibodies react with both the OC and OP-VDRE complexes in an indistinguishable manner (i.e., show quantitative similarities in the formation of supershift complexes).

required for the induction of VD3 responsive protein/DNA complexes at the OP-VDRE (Fig. 2). Therefore, to establish directly presence of the VDR in the OP-VDRE complex, we analyzed VDRE complexes detected with ROS 17/2.8 nuclear proteins, with additional VDR antibodies. Figure 3 shows that both OC- and OP-VDRE complexes are immunoreactive with the VD2F12 and 9a7γ antibodies. However, only the OC VDRE complex, but not the OP VDRE complex, reacts with the IVG8C11 antibody. We conclude that the OC- and OP-VDREs both interact with VDR/RXR heterodimers, but the observed differences in immunoreactivity with this panel of VDR antibodies unequivocally establish that these dimeric proteins are immunologically distinct.

To address if the order of addition of the reagents influences recognition of VDR/RXR heterodimers interacting with the OC- and OP-VDREs, we performed pre- and post-incubation experiments with the VDR antibody IVG8C11 added either prior to or after addition of radio-labelled VDREs. The results obtained in all instances were indistinguishable: we observed inhibition of VDR/RXR binding to the OC-VDRE and absence

of inhibition for the OP-VDRE (data not shown; see Figs. 1 and 3). Consistent with the observed inhibition of the VDR/RXR complex interacting with the OC-VDRE, the VDR antibody IVG8C11 recognizes an epitope near the DNA binding domain of the VDR. As inhibition of the VDR/RXR complex at the OC-VDRE also occurs after addition of DNA, it appears that the VDR epitope recognized by IVG8C11 is not masked by the mere presence of DNA. We also note that the IVG8C11 antibody recognizes the VDR independently of DNA in western blot and immunoprecipitation experiments (41,43). Thus, accessibility of the IVG8C11 antibody for the VDR epitope does not require contact between the VDR and the OC-VDRE. The striking observation that the VDR antibody IVG8C11 does not inhibit binding of VDR/RXR heterodimers to the OP-VDRE, appears to be related to specific DNA sequences in the OP-VDRE. The loss of epitope accessibility for the IVG8C11 apparently is mediated by the molecular restructuring of the VDR protein upon binding to the OP-VDRE.

We addressed the possibility that differential immunoreactivity of VDR/RXR heterodimers to the OC- and OP-VDREs is related to the presence of distinct members of the RXR family. Heterodimerization of different RXR members with the VDR could influence epitope accessibility of the VDR by steric hindrance or by inducing conformational modifications of the VDR. To address these possibilities we used monoclonal antibodies specific for RXRα and RXRß in gel shift immuno-assays, as ROS 17/2.8 cells express mRNAs for RXRα and RXRβ but not RXRγ (39). The results show (Fig. 3) that only a monoclonal antibody against RXRα, but not RXRβ, quantitatively reacts with the Vitamin D inducible complexes of both the OC- and OP-VDREs. Therefore, VDR/RXRα heterodimers are the predominant VDRE binding proteins for both the OC and OP genes in ROS 17/2.8 cells. This implies that differential immunoreactivity of the OC and OP VDRE complexes involves the same pair of hormone receptor proteins.

To verify these conclusions, we analyzed VDRE binding and immunoreactivity of VDR and RXRα proteins that were synthesized *in vitro* by coupled transcription/ translation using reticulocyte lysates (Fig. 4). The data indicate that VDR and RXRα together mediate complex formation at both the OC- and OP-VDREs. Also, when gel shift binding reactions and electrophoresis are performed in parallel, these OC- and OP-VDRE complexes formed from *in vitro* synthesized components have very similar electrophoretic mobilities when compared to VDRE complexes formed with native

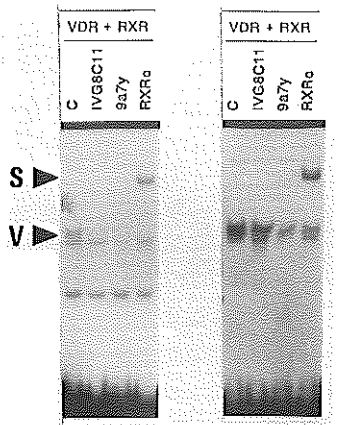

Figure 4

Differences in immunoreactivity are observed for in vitro transcribed/translated VDR and RXRα proteins which interact with the OC- and OP-VDREs. Gel shift incubations were performed with *in vitro* transcribed/translated VDR and RXRα in the presence of VD3 (10^{-8} M) using either OC- (left portion) or OP-VDRE (right portion) as probe. Immunoreactivity of the VDR/RXRα complexes (V) with the VDR antibody 9a7γ, the RXR antibody RXRα (S) and the VDR antibody IVG8C11 was investigated.

VDR/RXRα heterodimers from Vitamin D treated ROS 17/2.8 cells (data not shown).

Contribution of specific nucleotides to the conformational properties of VDR/RXRα heterodimers

The OC VDRE is composed of an imperfect direct repeat of two distinct steroid hormone half elements with a three nucleotide spacing (5'-GGGTGA NNN AGGACA), whereas the OP VDRE represents a perfect direct repeat of a different sequence motif (5'GGTTCA NNN GGTTCA). We tested the hypothesis that variations in the sequences of the steroid half-sites in the OC- and OP-VDREs directly influence immunoreactivity of the corresponding VD3 inducible complexes.

To evaluate the contribution of the OC and OP VDRE steroid half elements to recognition by the cognate proteins, we introduced a series of substitution mutations in the half-sites of the OC VDRE. The first mutant OP/OP contains several substitutions recreating the OP steroid half elements within the context of the OC VDRE (Table 2), whereas two other mutants contain unique mutations in either the distal (mutant OP/OC) or proximal (mutant OC/OP) steroid half element of the OC-VDRE. Figure 5 shows that each of these mutants compete with equal efficiency for the protein/DNA complexes interacting with the OC- and OP-VDREs. Thus, the mutations introduced into the OC-VDRE do not have significant rate-limiting effects on the interactions of VDRE binding proteins.

Immunoreactivity of VDR/RXRα heterodimers binding to this panel of

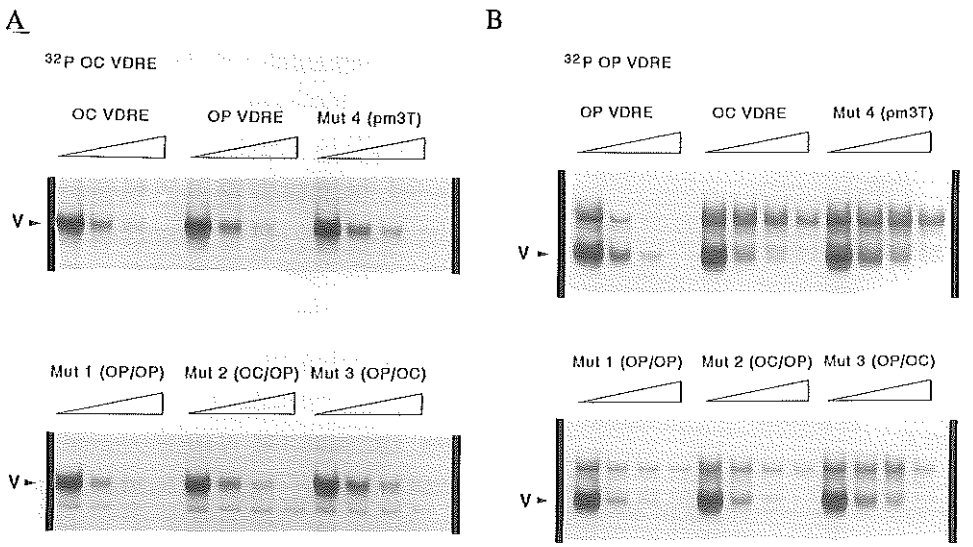

Figure 5

Competition analyses of mutant oligonucleotides containing basepair substitutions in VDRE sequences. Competition assays were performed with a fixed amount of nuclear protein from VD3 treated ROS 17/2.8 cells and the radio-labelled OC- (panel A) or OP-VDRE (panel B) oligonucleotides (0.5 nM). The titration experiments for each wildtype or mutant competitor oligonucleotide (as indicated above the lanes) was carried out by adding the unlabelled DNA fragments at the following concentrations: 0, 12, 25, 50 nM, respectively (from left to right). The oligonucleotides designated mut 1 OP/OP, mut 2 OC/OP, mut 3 OP/OC represent hybrid VDREs in which one or both of the hexameric steroid half elements of the OC-VDRE is replaced by the analogous sequences of the OP-VDRE, whereas pm 3T represent a point-mutant with a G to T substution at position 3 within the the proximal OC-VDRE hexamer (see Table 2 for further details).

substitution-mutants, using the VDR antibody IVG8C11 and RXR antibody RXRα,β,γ, is presented in Fig 6. The OP/OP mutant shows that replacement of the OC VDRE half sites by the steroid hormone half elements of the OP VDRE results in loss of immunoreactivity with the VDR antibody (Fig. 6). This finding establishes that the sequences determining differences in antibody reactivity between the OC and OP VDREs reside exclusively in the steroid hormone half sites, rather than in the flanking sequences (5', 3' or internal to the half-sites) of the OC and OP VDREs.

Strikingly, mutation of the proximal half site in the OC VDRE (mutant OC/OP) results in abrogation of VDR antibody (IVG8C11) recognition (Fig. 6). In contrast,

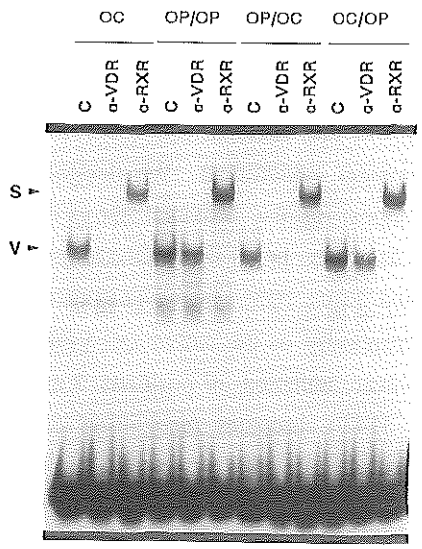

Figure 6

Oligonucleotidescontainingsubstitution-mutations within the OC-VDRE (see Table 2) were analyzed for immunoreactivity with the IV8C11 VDR antibody. Complexes were formed with nuclear extracts prepared from VD3 treated (10^{-8} M, 24 hr) ROS 17/2.8 cells and analyzed with the VDR antibody. Each oligonucleotide forms a VD3 inducible complex (V) and each of these complexes was tested in the absence (lane C) or presence of the IVG8C11 VDR antibody (α-VDR) and the RXRα,β,γ antibody (α-RXR).

mutation of the distal steroid half element (mutant OP/OC) in the OC VDRE does not alter VDR antibody reactivity. We conclude that integrity of the proximal half site of the OC VDRE is required for VDR antibody recognition. Furthermore, our results

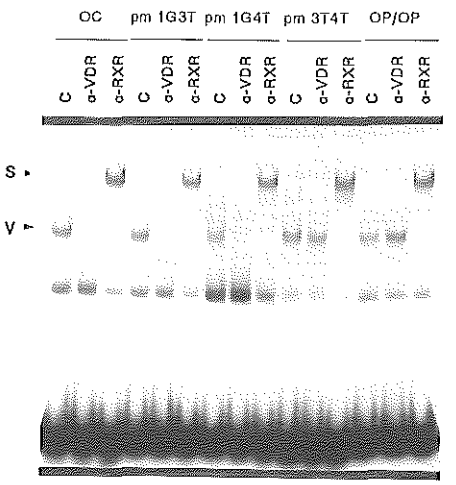

Figure 7

Point-mutational analysis of the OC-VDRE sequences shows that the central dinucleotide of the proximal hexameric steroid half element (5'-AGGACA, respectively, positions 1 to 6) mediates immunoreactivity. Several double-mutants were introduced in the proximal half site of the OC-VDRE (see Table 2) and the resulting mutant VDRE probes were incubated with nuclear extracts prepared from VD3-treated ROS 17/2.8 cells. The VD3-responsive complexes (V) for each probe are indicated by the arrowhead. The lanes designated "C" represent binding reactions in the absence of antibodies; the remaining lanes contain VDR and RXR antibodies as shown above the corresponding lanes. Similar to the wildtype OC-VDRE probe, the double-mutant probes pm 1G3T and pm 1G4T mediate complexes that immunoreact with the VDR antibody, but the double-mutant pm 3T4T does not immunoreact with the VDR antibody. All protein/DNA complexes show immunoreactivity ("supershift") (S) with the RXR antibody.

provide immunological evidence that the proximal half-site in the OC-VDRE interacts with the VDR.

The proximal half-sites of the OC- and OP-VDREs differ by exactly three nucleotides which are located at positions 1, 3 and 4 of the hexamer element (OC: 5'-AGGACA and OP: 5'-GGTTCA; nucleotide differences underlined). We designed a systematic series of double and single point-mutations of the proximal OC-VDRE hexamer. Analysis of this panel of hexamer mutants as probes in gel shift immuno-assays shows (Fig. 7) that double-mutations at positions 1 and 3 (pm 1G3T), as well as positions 1 and 4 (pm 1G4T) display the same VDR immunoreactivity as the native OC-VDRE. However, mutation of positions 3 and 4 (pm 3T4T) abrogates recognition of the IVG8C11 antibody for the VDR/RXRα complex. Thus, exchanging the internal purine-dinucleotide to a pyrimidine-dinucleotide at positions 3 and 4 prevents antibody accessibility to the epitope within the DNA binding domain of the VDR.

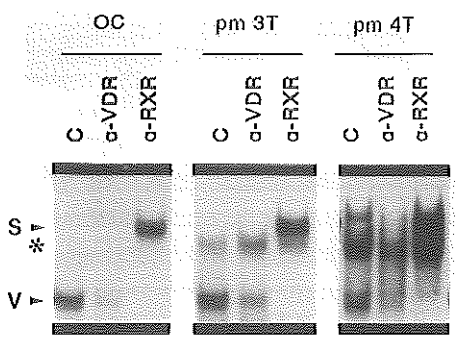

Figure 8

Two mutants with a single point-mutation in the proximal half site of the OC-VDRE (Table 2) were incubated with nuclear extracts prepared from VD3 treated ROS 17/2.8 cells and analyzed for immunoreactivity with both the VDR and RXR antibodies. Each oligonucleotide forms a VD3-responsive complex (V)(lane C), and immunoreactivity of each complex was analyzed using the antibodies indicated above the lanes. In contrast to the double-mutant probe pm 3T4T (Fig. 7), the immunoreactivity of the V complex observed with the single-mutant probes pm 3T and pm 4T (middle two panels) is more pronounced and resembles that of the wildtype OC VDRE probe (left panel).

To dissect the nucleotide-requirements of VDR antibody recognition further, we also performed gel shift immuno-assays with probes containing single point-mutations at either position 3 or 4 (pm 3T and pm 4T; Fig. 8). Notably, the third position within the VDRE half sites is thought to represent a key determinant for selective interactions with distinct VDRE binding proteins (14,15). However, single substitution of basepairs with the purine dinucleotide of the proximal VDRE hexamer at either position 3 (pm 3T) or 4 (pm 4T) does not cause this

abrogation of VDR immunoreactivity. Thus, although both nucleotides at positions 3 and 4 contribute together to immunoreactivity, neither alone is sufficient to mediate this effect.

DISCUSSION

In this study we characterized the Vitamin D inducible trans-activating complexes interacting with the OC- or OP-VDREs using gel shift immuno-assays with nuclear proteins from osseous cells. VD3 inducibility of VDRE complexes can be influenced by steroid and/or polypeptide hormones, as well as agents impinging on phosphorylation-dependent cell signalling mechanisms. In a series of related studies using osseous cells, we have analyzed the interactions of the VDR containing complexes with both the OC- and OP-VDREs using nuclear proteins from VD3 treated cells co-incubated with modulators of VDR function (43-45). For example, TGFβ1 and TGFβ2, each reduce the VD3 induced occupancy of both the OC and OP-VDREs (44). Similarly, co-incubation with okadaic acid, but not staurosporine, dexamethasone or 1β,25-dihydroxyvitamin D_3, results in a coordinate reduction of complex formation at both VDREs (45; unpublished observations). Thus, it appears that agents influencing VD3 mediated cell signalling pathways have parallel effects on the regulation of VD3 inducible complexes interacting with the OC-and OP-VDREs. Results obtained with a panel of monoclonal VDR and RXR antibodies establish that endogenous complexes interacting with the OC- and OP-VDREs quantitatively represent VDR/RXRα heterodimers. These findings provide a key addition to previous studies in which functional significance of homo- or heterodimerization of protein/DNA complexes at VDREs was pursued using different experimental strategies and biological systems (29-40). There appear to be discrepancies in the literature that relate to the potential for interactions of VDR homo- and heterodimers with the OC and OP VDREs. However, these apparent discrepancies may be attributable at least in part to the observation that VDR homo-dimers interact with lower affinity to VDREs than VDR/RXR heterodimers (14), as well as differences in experimental approaches.

Using endogenous nuclear proteins from ROS cells in which the VDR and RXR proteins are present at normal and physiologically regulated levels, our studies suggest

that the VDR containing complexes at both the OC and OP-VDREs have indistinguishable VDRE recognition specificity, are regulated in parallel by VD3 and VD3 antagonists, as well as are quantitatively composed of VDR/RXRα heterodimers. However, we find that VDR/RXRα heterodimers interacting with the OP- and OC-VDRE complexes are immunologically distinct, when analyzed with the monoclonal VDR antibody IVG8C11. The IVG8C11 antibody reacts with and inhibits formation of the VDR/RXRα complex at the OC-VDRE, but does not inhibit binding of VDR/RXRα heterodimers to the OP-VDRE.

By analyzing VDR immunoreactivity using a systematic series of point-mutations in the OC-VDRE, we show that the proximal half-site of OC-VDRE confers positive immunoreactivity with the VDR antibody. Thus, using the native OC-VDRE sequences and endogenously produced VDR/RXRα, this result provides a novel immunological line of evidence for the concept that the proximal VDRE half-site of the OC-VDRE interacts directly with the VDR, whereas the distal half-site interacts with RXRα (17,46-48). Furthermore, our results show that precisely two nucleotides at positions 3 and 4 of the proximal OC hexamer-element (OC: 5'-AGGACA versus OP: 5'-GGTTCA) determine the immunoreactivity of the VDR. Interestingly, the nucleotide at position 3 is thought to confer specificity for distinct VDR homo- and heterodimers (14,15). Here, we extend this observation by noting that positions 3 and 4 together influence the conformational properties of the same receptor heterodimer pair at two distinct VDREs, and distinguish between two apparent classes of VDR/RXRα heterodimers.

To understand the differences in dinucleotide-dependent immunoreactivity of the VDR/RXRα complexes, it is necessary to compare the location of the epitopes for VDR antibodies with known structural determinants in the DNA binding domains of steroid hormone receptors that specify recognition for the hexamer half-sites. Crystallographic data have revealed that the DNA binding domain of steroid hormone receptors represents a globular fold which contains two distinct Zn-nucleated substructures ("Zn-clusters")(4,49). The Zn-clusters are capable of adopting different conformations depending on the target DNA sequence. Read-out of DNA motifs occurs by the precise alignment of an amphiphatic α-helix with the major groove of DNA. Consistent with the importance of Zn cluster regions in VDR binding to DNA, this domain is extremely well conserved among mammalian species.

Studies by Freedman et al. (14) have provided compelling data for the concept that glu 42 of the VDR, which is located in the first Zn cluster (amino acids 24 to 44) of the VDR, contacts the nucleotide at position 3 in the VDRE hexamer. Our results show that position 3 is one of two basepairs required for differences in immunoreactivity of the OC- and OP-VDR/RXRα using the VDR antibody IVG8C11. Interestingly, the IVG8C11 antibody recognizes an epitope located between amino acids 57 and 164, which comprises the second Zn-cluster (amino acids 60 to 80) and portions of the flexible hinge-region towards the C-terminal ligand binding domain. In contrast, the VDR antibody 9a7γ which recognizes an epitope immediately adjacent to the second Zn cluster (between amino acids 89 and 105), reacts with VDR/RXRα heterodimers irrespective of the VDRE hexamer sequence. Taken together, these observations suggest that recognition of the central dinucleotide of the VDRE hexamer by the first Zn cluster may dictate the conformation of the second Zn cluster and/or the hinge-region. This central dinucleotide may influence the conformation of VDR/RXRα heterodimers because basepair differences in the VDRE hexamer translate into different steric and/or biochemical constraints on the molecular interactions between the recognition α-helix of the VDR and the VDRE hexamer. We also note that replacement of the OC hexamer dinucleotide (5'-GA) by the OP hexamer dinucleotide (5'-TT) may possibly affect the potential for DNA bending and/or kinking, or perhaps switch the orientation of the proximal hexamer relative to the distal hexamer of the VDRE.

Conformational modifications that are ligand dependent have been observed for many receptors, including the VDR (1-4). The results presented here provide the first evidence for conformational changes in VDR/RXRα complexes upon binding to distinct VDREs of the OC and OP genes. As the promoters of the OC and OP genes each interact with different trans-activating and repressor proteins, the conformational differences between OC- and OP-VDR/RXRα heterodimers may expose different protein/protein interaction surfaces. The trans-activating function of the VDR in the osteocalcin promoter involves potential contributions of TFIIB (50), MSX-related homeodomain proteins (51-54) and AP-1 activity (56). These transcription factors are capable of interacting with steroid hormone receptors (3) and may directly influence VDR/RXRα interactions with and/or trans-activation events at the OC-VDRE. Taken together, the conformationally distinct VDR/RXRα complexes binding to the OC- and OP-VDREs may reflect functional differences in VD3 mediated gene regulatory

mechanisms that control OC and OP gene transcription in response to physiological signals relevant to skeletal homeostasis.

ACKNOWLEDGMENTS

We thank Rajesh Desai and Jean Prahl for stimulating discussions, as well as Pierre Chambon and Wesley Pike for generously providing RXR and VDR (9a7γ) antibodies, respectively. These studies were supported by grants from NIH (AR-33920, AR-39588 and DK-46721), as well as the Netherlands Organization for Scientific Research (NWO; project no. 900-541-131).

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CHAPTER 8

GENERAL DISCUSSION

The aim of the study described in this thesis was to assess the significance of the locally produced growth factors transforming growth factor ß (TGFß), bone morphogenetic protein-2 (BMP-2) and insulin-like growth factor-I (IGF-I) for the action of the systemic factor 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) on bone. We show that locally produced factors affect several aspects of 1,25-$(OH)_2D_3$-regulated bone metabolism. First, we studied the effect of TGFß on vitamin D receptor (VDR) level and the relevance of modulation of VDR level for the action of 1,25-$(OH)_2D_3$ on osteocalcin and osteopontin expression and on 24-hydroxylase activity in the bone forming cell, the osteoblast. Secondly, we studied the effects of locally produced factors on stimulation of bone resorption by systemic factors and studied their presence and distribution in bone matrix. Finally, we studied the osteocalcin and osteopontin promoter regions more closely by investigating the significance of sequence variation in the vitamin D response elements (VDREs) for the composition of hormone receptor complexes recognizing the VDREs.

Regulation of vitamin D receptor level and 1,25-$(OH)_2D_3$ responses by TGFß

Transforming growth factor ß (TGFß), which is the most extensively investigated factor in this study induced an increase in vitamin D receptor (VDR) level in osteoblast-like cells of various origin. Based on this result and the general concept concerning the relationship between receptor level and hormone action we hypothesized that TGFß increases the biological responses to 1,25-$(OH)_2D_3$ in these cells. However, the 1,25-$(OH)_2D_3$-stimulated expression of two bone matrix proteins, osteocalcin and osteopontin, appeared to be inhibited after TGFß preincubation-induced up-regulation of the VDR. These observations clearly demonstrated a dissociation between the effect of TGFß on VDR level and its effect on two biological responses to 1,25-$(OH)_2D_3$ in osteoblast-like cells. Similarly, for glucocorticoids, a dissociation has been shown between regulation of receptor level and biological response (1). In contrast, previously a relationship between VDR level and several biological responses, including osteocalcin synthesis, inhibition of collagen synthesis and, especially, 24-hydroxylase activity, has been shown (2-5). To investigate the significance of regulation of VDR level more thoroughly, the effect of TGFß-induced increase in VDR level on the induction of 24-hydroxylase activity by 1,25-$(OH)_2D_3$ was examined. A direct relationship appeared to exist between regulation of VDR level and induction of 24-hydroxylase activity by 1,25-$(OH)_2D_3$, not only after regulation of VDR level by TGFß, but also after modulation of VDR level by other

means, including medium change, PTH and EGF incubation (Chapter 4). In contrast to induction of 24-hydroxylase activity, for other biological responses, VDR level modulation may not be the primary mechanism through which these effects (e.g. osteocalcin and osteopontin expression) are regulated. Additional effects may compensate the effect of changes in VDR level or even overrule this effect. Therefore, the inhibitory effect of TGFß on the 1,25-$(OH)_2D_3$ enhancement of osteocalcin and osteopontin was examined in more detail. In Figure 1 we show that TGFß causes its inhibitory effect on osteocalcin and osteopontin expression (Chapters 2 and 3) by the reduction of binding of nuclear protein complexes, containing VDR and RXRα (Chapter 7), to the vitamin D response elements (VDREs) in the osteocalcin and osteopontin promoter regions (Chapter 3). Because both the osteocalcin and osteopontin promoter regions contain binding sites for activator protein-1 (AP-1), which is a dimer of Fos and Jun, and both TGFß and 1,25-$(OH)_2D_3$ have been shown to modulate the expression of the fos and jun family of proto-oncogenes in osteoblasts (6-9), we also examined the effects of

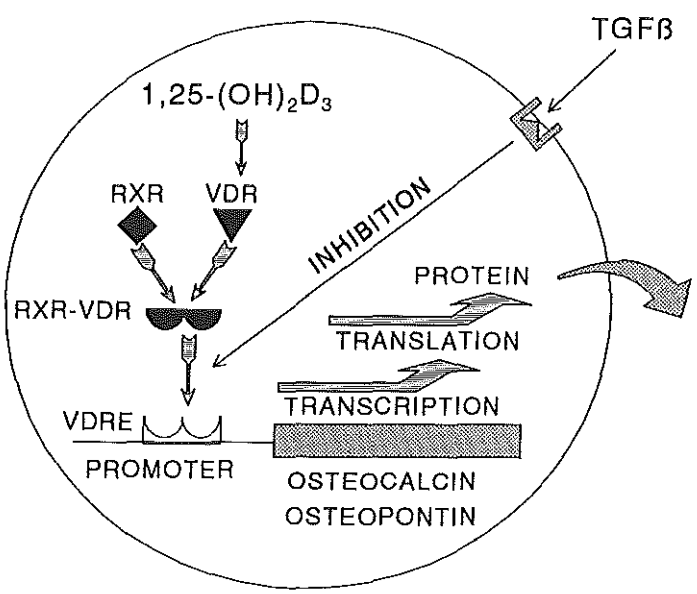

Figure 1

Model for the inhibitory effect of TGFß on the 1,25-$(OH)_2D_3$-induced binding of VDR/RXRα complexes to the vitamin D response elements of the osteocalcin and osteopontin genes, which leads to a reduction of 1,25-$(OH)_2D_3$-stimulated osteocalcin and osteopontin expression.

these agents on binding of nuclear protein complexes to an AP-1 consensus oligonucleotide. Our results (Chapter 3) indicate that increased levels of AP-1 binding may contribute to TGFß inhibition of osteocalcin gene transcription.

Data obtained by Pirskanen et al. (10) argue against a similar mechanism in the human MG 63 cells. They did not observe a direct inhibitory effect of TGFß on the 1,25-$(OH)_2D_3$-induced binding of nuclear protein complexes to the VDRE of the human osteocalcin gene. Jääskeläinen et al. (11) have recently shown that the MG 63 endogenous nuclear protein complex recognizing the human osteocalcin VDRE contains the VDR, but not RXRα. At this moment, the exact composition of the 1,25-$(OH)_2D_3$-induced complex recognizing the human osteocalcin VDRE remains unknown. The difference in the composition of the rat and human nuclear protein complexes binding their cognate VDREs may account for the differences in the mechanisms by which TGFß exerts its inhibitory effects on 1,25-$(OH)_2D_3$-stimulated osteocalcin expression. We (Chapter 7) and others (12-15) have provided evidence that the VDR binds the proximal half site and RXR the distal half site of the VDRE. Therefore, and because Jääskeläinen et al. (11) indicated that the complex recognizing the human osteocalcin VDRE differs in the accessory factor component (not RXRα), it is remarkable that the rat osteocalcin VDRE and human osteocalcin VDRE (Table 1) are identical in the distal half site, and differ at two nucleotides (the 1st and 4th) in the proximal half site. From the present study it remains unclear, whether there indeed exist species differences in the nuclear protein complexes binding the human and the rat osteocalcin VDRE and in the mechanisms of inhibition of 1,25-$(OH)_2D_3$-stimulated osteocalcin production by TGFß in human and rat osteoblast-like cells. However, the present study demonstrated a discrepancy between TGFß-induced VDR up-regulation and inhibition of 1,25-$(OH)_2D_3$-induced

Table 1

Species differences in the VDREs of the osteocalcin gene promoter. The nucleotides within the half sites that differ from the rat OC VDRE are underlined.

	osteocalcin VDREs
rat	G G G T G A nnn A G G A C A
human	G G G T G A nnn <u>G</u> G G <u>G</u> C A
mouse	G G G <u>C A</u> A nnn A G G A C A

osteocalcin expression irrespective of species.

Besides this direct effect of TGFß on VDR/RXR interaction with the VDRE in the promoter region of the osteocalcin and osteopontin genes, the observed increase in 1,25-$(OH)_2D_3$ -induced 24-hydroxylase activity as a result of up-regulation of VDR by TGFß may play an indirect inhibitory role. 24-Hydroxylation is the initial step in a more extensive C24-oxidation pathway that leads to the degradation and elimination of 1,25-$(OH)_2D_3$. The relationship between VDR level and this response may reflect a negative regulatory feedback loop at target tissue/cell level in the action of 1,25-$(OH)_2D_3$. As is shown in Figure 2, TGFß may both directly (Chapter 3) and indirectly (hypothesis) inhibit the stimulation of expression of the osteocalcin and osteopontin genes by 1,25-$(OH)_2D_3$. Directly (arrow 1), TGFß inhibits the 1,25-$(OH)_2D_3$-stimulated osteocalcin and osteopontin expression by reducing the binding of VDR/RXRα heterodimeric complexes to the VDREs in the promoter regions of

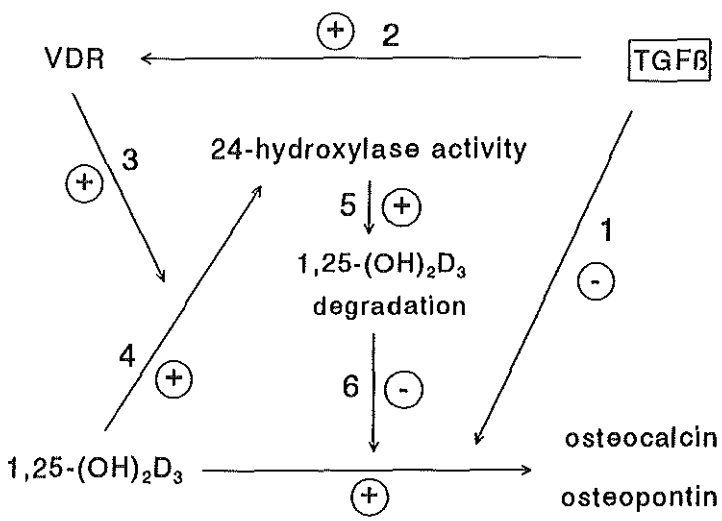

Figure 2

Model for a direct and indirect mechanism of TGFß inhibition of 1,25-$(OH)_2D_3$-stimulated osteocalcin and osteopontin expression. 1) Directly, TGFß has been shown to reduce the 1,25-$(OH)_2D_3$-induced binding of nuclear protein complexes to the osteocalcin and osteopontin vitamin D responsive elements. Indirectly, 2) TGFß causes an up-regulation of VDR level, which leads to 3 and 4) enhancement of 1,25-$(OH)_2D_3$-stimulated 24-hydroxylase activity. 5) Enhanced 24-hydroxylase activity leads to the degradation of 1,25-$(OH)_2D_3$. 6) A reduced amount of 1,25-$(OH)_2D_3$ causes a reduced response of osteocalcin and osteopontin to 1,25-$(OH)_2D_3$.

osteocalcin and osteopontin (Chapter 3). Indirectly, the increase in VDR level induced by TGFß (arrow 2) results in an enhanced induction of the 24-hydroxylase activity by 1,25-$(OH)_2D_3$ (arrows 3 and 4). Subsequently, the amount of biologically active 1,25-$(OH)_2D_3$ is diminished (arrow 5), thereby reducing the 1,25-$(OH)_2D_3$ stimulation of osteocalcin and osteopontin expression (arrow 6). This indirect effect may only play a prominent role in MG 63 cells. In this cell line, we have shown the effects of TGFß both on 1,25-$(OH)_2D_3$-stimulated osteocalcin and 1,25-$(OH)_2D_3$ induction of 24-hydroxylase activity. In ROS 17/2.8 cells we showed the effects of TGFß on 1,25-$(OH)_2D_3$ enhancement of osteocalcin and osteopontin expression, but not its effects on the induction of 24-hydroxylase activity, because 1,25-$(OH)_2D_3$-induced 24-hydroxylase activity appeared hardly detectable in ROS 17/2.8 cells (data not shown). This indicates that regulation of 24-hydroxylase activity does not play a major role in the inhibitory effect of TGFß on 1,25-$(OH)_2D_3$-stimulated osteocalcin and osteopontin expression in ROS 17/2.8 cells.

Growth factor specificity in the regulation of 1,25-$(OH)_2D_3$ action on osteocalcin and osteopontin expression

The other two growth factors studied for their effect on 1,25-$(OH)_2D_3$ action were IGF-I and BMP-2. The results obtained with these growth factors clearly indicate that the responses are growth factor specific. The inhibitory effect observed with TGFß on 1,25-$(OH)_2D_3$-enhancement of osteocalcin and osteopontin expression appeared to be specific for TGFß. In contrast to $TGFß_1$ and $TGFß_2$, BMP-2 did not affect 1,25-$(OH)_2D_3$ enhanced osteocalcin synthesis as well as osteocalcin mRNA expression, whereas it stimulated the 1,25-$(OH)_2D_3$ enhancement of osteopontin mRNA expression (Chapter 5). IGF-I did not affect basal and 1,25-$(OH)_2D_3$-stimulated osteocalcin synthesis. The 1,25-$(OH)_2D_3$-induced increase in osteocalcin and osteopontin mRNA expression was not significantly affected by IGF-I (Chapter 5). However, gel shift mobility assays revealed similarities between TGFß and BMP-2 in the regulation of 1,25-$(OH)_2D_3$ inducible binding of nuclear protein complexes to the osteocalcin and osteopontin VDREs. Like TGFß, BMP-2 reduced the binding of VDR/RXRα complexes to these VDREs. IGF-I slightly reduced binding of VDR/RXRα complexes to the osteopontin VDRE, but did not affect binding to the osteocalcin VDRE. These observations demonstrate a dissociation between regulation of osteocalcin and osteopontin expression and regulation of the binding of

VDR/RXRα complexes to the osteocalcin and osteopontin VDREs by BMP-2 and IGF-I. This indicates that additional mechanisms, that compensate and overrule the effect of reduced binding of VDR/RXRα complexes to the VDREs, e.g. binding of nuclear proteins to other regulatory sites in the promoter region or effects mRNA on stabilization, play a role in the biological response to these factors.

Regulation of bone resorption by locally produced growth factors

In Chapter 6 the effect of TGFß on bone resorption was studied. Osteocalcin and osteopontin have been suggested to play a role in bone resorption, osteocalcin by its involvement in the recruitment and/or differentiation of osteoclasts (16,17) and osteopontin by the attachment of osteoclasts to bone (18,19). In view of the observed effects of TGFß on 1,25-$(OH)_2D_3$-induced osteocalcin and osteopontin expression in rat and human osteoblasts (Chapters 2 and 3) we were interested in the effect of TGFß on 1,25-$(OH)_2D_3$ enhancement of bone resorption. The effects of TGFß were examined in a fetal mouse bone resorption system. Similar to its effect on 1,25-$(OH)_2D_3$-enhanced osteocalcin and osteopontin expression in MG 63 and ROS 17/2.8 cells, TGFß appeared to inhibit 1,25-$(OH)_2D_3$ stimulation of bone resorption (Chapter 6), thereby supporting the hypothesis that osteocalcin and osteopontin play a role in bone resorption. However, in fetal mouse long bone cultures 1,25-$(OH)_2D_3$ itself appeared to reduce osteocalcin levels, this in contrast to observations in non-fetal rat and human osteoblast-like cells. Also, in the non-fetal mouse osteoblast-like cell line MC3T3, 1,25-$(OH)_2D_3$ reduced osteocalcin levels in medium and cell extracts (Chapter 6), indicating that the reduction we observed in osteocalcin levels in medium of fetal mouse long bones after incubation with 1,25-$(OH)_2D_3$ is due to species difference. In relation with our data presented in Chapter 7, a possibility can be that these species differences are due to a dinucleotide difference in the distal half site the vitamin D response element in the promoter of rat/human and the mouse osteocalcin gene (Table 1). However, additional elements in the mouse osteocalcin promoter may play an important role in the inhibitory effect of 1,25-$(OH)_2D_3$.

Despite the difference in regulation of the osteocalcin gene between mouse and rat/human *in vitro*, Gundberg et al. (20) showed in *in vivo* experiments that osteocalcin level is increased in the serum of mice after 1,25-$(OH)_2D_3$ administration, thereby demonstrating a discrepancy between osteocalcin regulation *in vitro* and *in vivo*. The fact that *in vivo* mouse osteocalcin levels are increased by 1,25-$(OH)_2D_3$, does not exclude the possibility that osteocalcin may indeed play a role in bone

resorption. In addition, data of Desbois et al. (21), who showed that osteocalcin deficiency in mice leads to increased bone formation, may also be suggestive for a role of this bone matrix protein in bone resorption in mice. Figure 3 represents a model for the role of TGFß in bone remodelling in relation to its effects on bone formation, 1,25-$(OH)_2D_3$-stimulated bone resorption, osteocalcin and osteopontin expression. The primary effect of TGFß is stimulation of bone formation, both by directly stimulating bone formation (22-25), but also by reducing bone resorption (Chapter 6). The proposed intermediary role of osteocalcin and osteopontin in the TGFß-inhibition of 1,25-$(OH)_2D_3$-stimulated bone resorption is based on the postulated roles of these proteins in bone resorption (16-19), together with the TGFß inhibition of 1,25-$(OH)_2D_3$-stimulated osteocalcin and osteopontin expression, and the data of Gundberg et al, and Desbois et al mentioned above. This despite the fact that the data obtained with the *in vitro* mouse bone resorption model do not directly provide evidence for a role of osteocalcin in bone resorption (Chapter 6). This, however, can be due to the fact that osteocalcin is involved in aspects of the bone resorption process (e.g. recruitment, differentiation of osteoclasts) which do not play a prominent role is this bone resorption model. As shown in Figure 3, during bone resorption bone matrix is degraded and latent TGFß that is present in the bone matrix can be released and activated during bone resorption (26,27), which in turn leads to inhibition of 1,25-$(OH)_2D_3$-stimulated bone resorption and to stimulation of bone formation. This model provides a target tissue feedback mechanism by which via close interaction between a bone cell-derived and bone matrix-stored growth factor and a calciotropic hormone bone metabolism is controled.

The other two growth factors studied were IGF-I and BMP-2. IGF-I had, similar to TGFß, an inhibitory effect on 1,25-$(OH)_2D_3$-stimulated bone resorption. In contrast, BMP-2 caused an enhancement of 1,25-$(OH)_2D_3$-stimulated bone resorption. The results obtained with these growth factors clearly indicate that the regulation of 1,25-$(OH)_2D_3$ -enhanced bone resorption is growth factor specific (Chapter 5). These data are thereby in line with the data obtained with 1,25-$(OH)_2D_3$-induced osteocalcin and osteopontin expression, which also demonstrated growth factor specific interactions. In addition, data presented in Chapter 6 not only demonstrate that 1,25-$(OH)_2D_3$-stimulated bone resorption is regulated in a growth factor specific manner, but also that the growth factor interactions are hormone specific. For example, TGFß and BMP-2 only affected 1,25-$(OH)_2D_3$-, but not PTH-stimulated bone resorption, whereas IGF-I affected both 1,25-$(OH)_2D_3$ and PTH-stimulated

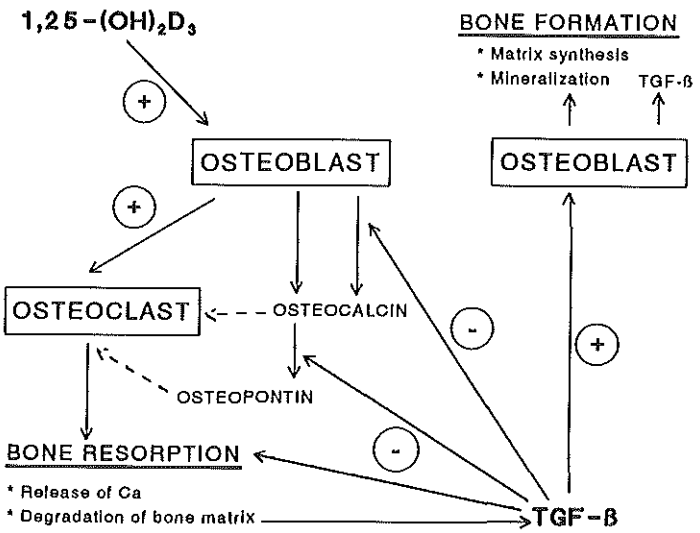

Figure 3

Model for the role of TGFß in bone metabolism. TGFß has been reported to enhance bone formation by a stimulatory effect on the osteoblast. Furthermore, TGFß inhibits 1,25-$(OH)_2D_3$-stimulated bone resorption. The bone matrix proteins osteocalcin and osteopontin have been proposed to play a role in bone resorption by affecting the recruitment, differentiation and attachment of osteoclasts (dotted lines). TGFß inhibits the 1,25-$(OH)_2D_3$-stimulated expression of osteocalcin and osteopontin in the osteoblast and may thereby exert its effect on 1,25-$(OH)_2D_3$-stimulated bone resorption. During bone resorption, bone matrix is degraded and factors stored in the bone matrix, including TGFß, are released.

bone resorption.

Taken together, these data implicate that growth factors are involved in the control of the extent of bone resorption in response to 1,25-$(OH)_2D_3$ and/or PTH. To gain insight into the localization of these growth factors in the bone matrix, the presence and distribution of TGFß type 2, TGFß type 3, BMP-2 and IGF-I in adult human bone were examined. Immunohistochemical analysis revealed that the presence of these growth factors is restricted to specific sites and that they are not diffusely distributed. Thus, the amount and type of growth factor present in the bone matrix may eventually cause less or more profound resorption in response to 1,25-$(OH)_2D_3$ and/or PTH at particular sites. For example, 1,25-$(OH)_2D_3$-induced bone resorption at a TGFß-positive site may be limited whereas at a TGFß-negative site

resorption can go on. The proposed model requires that growth factors should be released from the bone matrix during resorption and must still be biologically active or in a configuration to be activated. This seems not unlikely because TGFß is stored in a latent form in the bone matrix (26,28) and IGF-I binds to IGF-BPs which are capable of binding to the extracellular matrix (29,30). These features can have protective effects for the growth factors which may result in the eventual release of active growth factors. This is supported by the *in vitro* observation that several factors that stimulate bone resorption at the same time enhance TGFß activity in the culture medium (26,27). This indeed suggests that TGFß, but possibly also the other growth factors, may serve as a local feedback regulatory mechanism for bone resorption.

The significance of a dinucleotide difference between vitamin D response elements for the conformation of nuclear protein complexes binding to these elements

In Chapter 7, we have shown that a dinucleotide difference in the proximal half site of the rat osteocalcin VDRE and mouse osteopontin VDRE results in a different immunoreactivity of the nuclear protein complexes binding to these VDREs, which is probably due to a difference in the conformation of the VDR. Conformational modifications that are ligand dependent have been observed for many receptors, including the VDR. The results presented here provide the first evidence for conformational changes in VDR/RXRα complexes upon binding to distinct VDREs of the osteocalcin and osteopontin genes. As the promoters of the osteocalcin and osteopontin genes each interact with different trans-activating and repressor proteins, the conformational differences between osteocalcin- and osteopontin-VDR/RXRα heterodimers may expose different protein/protein interaction surfaces. The trans-activating function of the VDR in the osteocalcin promoter involves potential contributions of transcription factor IIB (TFIIB), MSX-related homeodomain proteins and AP-1 activity. These transcription factors are capable of interacting with steroid hormone receptors and may directly influence VDR/RXRα interactions with and/or trans-activation events at the osteocalcin VDRE. Taken together, the conformationally distinct VDR/RXRα complexes binding to the osteocalcin and osteopontin VDREs may reflect functional differences in 1,25-$(OH)_2D_3$ mediated gene regulatory mechanisms. Thereby, this may have physiological implications for the differential regulation of these two genes in the control of skeletal homeostasis.

In view of our data on 1,25-$(OH)_2D_3$ regulation of osteocalcin in mice and rat

(Chapter 6), one has to consider that the observed conformation differences can be due to species differences. The VDREs that were compared were from rat (osteocalcin) and mouse (osteopontin) origin. Very recently, in the rat osteopontin promoter region two functional VDREs have been identified (31). Comparison of the transcriptionally most potent (8-fold induction) rat osteopontin VDRE, AGGTCAnnnGGTTCA, and the mouse osteopontin VDRE, GGTTCAnnnGGTTCA, demonstrates that the proximal, VDR-binding, half-sites are identical. Therefore, it is unlikely that the observed conformational differences in the VDR/RXR complex are due to species differences but more likely are due to intrinsic differences in the osteocalcin and osteopontin VDREs.

Conclusions

Taken together, this study shows that the locally produced factors TGFß, BMP-2 and IGF-I have important effects on the regulation of bone metabolism by the systemic factor 1,25-$(OH)_2D_3$ and do not just serve as mediators through which 1,25-$(OH)_2D_3$ exerts its action. This is demonstrated by the effects of TGFß, BMP-2 and IGF-I on 1,25-$(OH)_2D_3$-stimulated osteocalcin and osteopontin expression and bone resorption. More general, this may imply that hormone action depends on the locally produced factors present in target tissues. The modulation of hormone action by locally produced factors may occur at several levels in the target cell, e.g. from the regulation of hormone receptor level to the regulation of the binding of nuclear proteins to regulatory sites in the promoter region of genes.

Suggestions for further research

To gain more insight into the specific roles of locally produced factors in the modulation of vitamin D action and the mechanism of action of locally produced factors further research is necessary. A major concern in our study is the observation that species differences exist in the biological responses to locally produced factors and 1,25-$(OH)_2D_3$. Our studies revealed that in *in vitro* experiments an opposite effect is observed in the regulation of osteocalcin synthesis by 1,25-$(OH)_2D_3$ between rat/human and mouse. Therefore, it would be interesting to compare the compositions of nuclear protein complexes that bind the rat, human and mouse osteocalcin VDREs. Despite the similarity in the effects of TGFß and 1,25-$(OH)_2D_3$ in rat osteoblast-like (ROS 17/2.8) and human osteoblast-like (MG 63) cells on osteocalcin synthesis, there are indications that the mechanisms whereby the

inhibitory effect of TGFß on 1,25-$(OH)_2D_3$ enhancement of osteocalcin synthesis is exerted, differ (cf. page 168). Therefore it is important that the experiments on the effects of locally produced factors on 1,25-$(OH)_2D_3$ are performed in a human model and that the mechanisms whereby local factors regulate 1,25-$(OH)_2D_3$ action are studied in the human system.

In this Chapter (cf. page 169) we proposed a model for the possible role for 24-hydroxylase activity regulation in the inhibitory effect of TGFß on 1,25-$(OH)_2D_3$-stimulated osteocalcin and osteopontin expression. Further studies are needed to determine whether this mechanism indeed plays a role in the inhibitory action of TGFß on 1,25-$(OH)_2D_3$-stimulated osteocalcin and osteopontin expression.

The most extensively studied factor in this thesis was TGFß. Additionally, also the effects of BMP-2 and IGF-I were studied on 1,25-$(OH)_2D_3$-stimulated osteocalcin and osteopontin expression and bone resorption. These factors also appeared to affect these parameters of bone metabolism. However, only modest effects were observed in the regulation of osteocalcin and osteopontin expression by BMP-2 and IGF-I. To gain further insight in their effects on these genes prolonged incubations may be necessary to determine biologically relevant effects and the function of these factors in bone metabolism.

Furthermore, the exact function of osteocalcin and osteopontin in the regulation of bone remodelling is still unknown. To gain more insight into their role in the regulation of the bone remodelling process, it would be interesting to study their function more thoroughly.

Finally, eventually *in vivo* studies will be necessary to reveal whether the effects we have observed in *in vitro* studies play indeed an important role in the regulation of bone remodeling *in vivo*.

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CHAPTER 9

SUMMARY

SUMMARY

The steroid hormone 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) plays a principal role in calcium homeostasis and skeletal metabolism, and influences expression of genes related to establishment and maintenance of the bone cell phenotype. 1,25$(OH)_2D_3$ exerts its primary effect by binding to its receptor, the vitamin D receptor (VDR), which belongs to the steroid/retinoid/thyroid hormone receptor superfamily and acts via binding to distinct vitamin D response elements (VDREs) in the promoter region of several genes.

Besides systemic calciotrophic factors, like 1,25-$(OH)_2D_3$, a wide variety of locally produced factors has been shown to affect bone metabolism. Systemic factors have been demonstrated to modulate the production of local factors thereby regulating bone formation and resorption. However, the presence of locally produced factors may also have implications for the biological response to systemic factors. Therefore, it is conceivable that local factors may interfere in the action of calciotrophic hormones in bone. The factors transforming growth factor ß (TGFß), bone morphogenetic protein-2 (BMP-2) and insulin-like growth factor-I (IGF-I) are produced by bone cells and are stored in the bone matrix. The aim of the study described in this thesis was to assess the significance of the locally produced growth factors TGFß, BMP-2 and IGF-I for the action of the systemic factor 1,25-$(OH)_2D_3$ on bone.

First, the effect of TGFß, which is the most extensively investigated factor in this study, on the VDR level in bone forming, osteoblast-like cells was determined. In osteoblasts of various origin TGFß caused a comparable increase in VDR level (Chapter 2). To assess the significance of the TGFß-induced increase in VDR level for the action of 1,25-$(OH)_2D_3$ on osteocalcin and osteopontin expression in these cells, cells were *pre*incubated with TGFß (causing an increase in VDR level) and subsequently incubated with 1,25-$(OH)_2D_3$. Osteocalcin and osteopontin are non-collagenous bone matrix proteins secreted by osteoblasts and have been suggested to play a role in bone resorption, osteocalcin by its involvement in the recruitment and/or differentiation of the bone resorbing osteoclasts and osteopontin by the attachment of osteoclasts to bone. TGFß preincubation potently inhibited subsequent 1,25-$(OH)_2D_3$ stimulation of osteocalcin and osteopontin expression in osteoblast-like cells (Chapters 2 and 3). This observation demonstrates a dissociation between regulation of VDR level and modulation of two 1,25-$(OH)_2D_3$ biological responses by

TGFß in osteoblast-like cell lines.

In Chapter 3, we investigated the mechanism by which TGFß inhibits 1,25-$(OH)_2D_3$ enhanced expression of the osteocalcin and osteopontin genes. ROS 17/2.8 osteoblast-like cells, in which both genes are expressed, were transfected with reporter constructs driven by native (*i.e.* wild-type) rat osteocalcin and mouse osteopontin promoters. TGFβ abrogated the 1,25$(OH)_2D_3$ enhanced transcription of both the osteocalcin and osteopontin genes. The inhibitory TGFβ response for each requires VDRE sequences, although there are additional contributions from proximal basal regulatory elements. These transcriptional effects were further investigated for contribution of the *trans*-activating factors, which interact with osteocalcin and osteopontin VDREs, involving the VDR and retinoic X receptor (RXR). Gel mobility shift assays showed that TGFß significantly reduces induction of the heterodimeric VDR/RXR complexes in 1,25$(OH)_2D_3$ treated ROS 17/2.8 cells, which was not accounted for by changes in VDR protein levels. We also showed that activator protein-1 activity, which is up-regulated by TGFβ, may potentially contribute to inhibitory effects of TGFβ on basal osteocalcin transcription.

A response that has formerly been shown to be coupled to VDR level is 24-hydroxylase activity. This enzyme initiates the C24-oxidation pathway for side-chain oxidation, followed by cleavage and ultimate metabolic clearance of 1,25-$(OH)_2D_3$ and its precursor 25-$(OH)D_3$. To investigate the significance of regulation of VDR level more thoroughly, in Chapter 4 the effect of TGFß-induced increase in VDR level on the induction of 24-hydroxylase activity by 1,25-$(OH)_2D_3$ was examined. We showed that after preincubation with TGFß, which causes an increase in VDR level, the 1,25-$(OH)_2D_3$ induction of 24-hydroxylase activity is further stimulated by TGFß in two osteoblast-like cell lines. Using several other means to regulate VDR level, we provided evidence that a tight coupling exists between VDR level and induction of the C24-oxidation pathway by 1,25-$(OH)_2D_3$. As C24-oxidation is involved in the catabolic pathway of 1,25-$(OH)_2D_3$, the coupling of VDR level to the degradation of 1,25-$(OH)_2D_3$ may provide an important regulatory feedback mechanism in the action of 1,25-$(OH)_2D_3$ at target tissue/cell level.

In Chapter 5, the effects of two other locally produced growth factors, bone morphogenetic protein-2 (BMP-2) and insulin-like growth factor-I (IGF-I) on the action of 1,25-$(OH)_2D_3$ on osteocalcin and osteopontin expression were compared with the effects of TGFß. Differential effects of these three growth factors were found on the 1,25-$(OH)_2D_3$-stimulated osteocalcin and osteopontin expression and on the

binding of nuclear protein complexes to the osteocalcin and osteopontin VDREs. Thus, the results obtained with these growth factors clearly indicate that the interaction with 1,25-$(OH)_2D_3$ is growth factor specific.

In Chapter 6, we investigated the effects of TGFß, BMP-2 and IGF-I on the bone resorptive action of the systemic factors 1,25-$(OH)_2D_3$ and parathyroid hormone (PTH). Our results show that TGFß inhibits 1,25-$(OH)_2D_3$-enhanced bone resorption, but not PTH-enhanced bone resorption, whereas IGF-I inhibits both PTH- and 1,25-$(OH)_2D_3$-enhanced bone resorption. BMP-2 had, depending on its concentration, a stimulatory effect on 1,25-$(OH)_2D_3$-enhanced bone resorption (10-100 ng/ml), or had at 1000 ng/ml a strong stimulatory effect on basal bone resorption and no further effect on 1,25-$(OH)_2D_3$ and PTH action. These data confirm the growth factor specificity of the effects of TGFß, BMP-2 and IGF-I on 1,25-$(OH)_2D_3$ action described in the previous Chapter. Furthemore, these data show that the growth factor effects are also hormone specific. In addition, the effects of local and systemic factors on osteocalcin and collagenase were studied as possible mediators involved in the regulation of bone resorption. Remarkably, 1,25-$(OH)_2D_3$ had, in contrast to its stimulatory effect on bone resorption, an inhibitory effect on the osteocalcin protein levels in fetal mouse long bones. In addition, this inhibitory effect of 1,25-$(OH)_2D_3$ was also observed in the mouse osteoblast-like cell line MC3T3. These observations point to species specific regulation of osteocalcin by 1,25-$(OH)_2D_3$. 1,25-$(OH)_2D_3$ did not affect collagenase activity.

Analysis of the distribution of the locally produced growth factors in the bone matrix demonstrated that in adult human bone the presence of TGFß type 2, TGFß type 3, BMP-2 and IGF-I appears to be restricted to specific areas in bone, and are not diffusely distributed. This observation may implicate that locally produced factors control the response to systemic factors at particular sites in bone. Thus, the amount and type of growth factor present in the bone matrix may eventually cause less or more profound resorption in response to 1,25-$(OH)_2D_3$ and/or PTH at particular sites.

Sequence variation between the osteocalcin and osteopontin VDRE steroid hormone half-elements provides the potential for recognition by distinct hormone receptor homo- and heterodimers. In Chapter 7, we showed that 1,25-$(OH)_2D_3$ inducible complexes interacting with the osteocalcin and osteopontin VDREs represent two distinct heterodimeric complexes, each composed of the VDR and RXRα. We found that two internal nucleotides of the proximal osteocalcin VDRE half-site

(5'-AGGACA) determine differences in VDR immunoreactivity. The results in Chapter 7 provide evidence for polarity of VDR/RXRα binding to osteocalcin and osteopontin VDREs, with the proximal half-elements being recognized by the VDR, and further suggest that the DNA binding domain of the VDR adopts different protein conformations when contacting distinct VDREs. This may have physiological implications for the differential regulation of these two genes in the control of skeletal homeostasis.

Taken together, this study shows that the locally produced factors TGFß, BMP-2 and IGF-I have important effects on the action of the systemic factor 1,25-$(OH)_2D_3$ and do not just serve as mediators through which 1,25-$(OH)_2D_3$ exerts its action. More general, this type of interactions may also play an important role in the action of other hormones.

SAMENVATTING

Het steroid hormoon 1,25-dihydroxyvitamine D_3 (1,25-$(OH)_2D_3$) speelt niet alleen een belangrijke rol in de calciumhuishouding en het botmetabolisme, maar beïnvloedt tevens de expressie van genen, die betrokken zijn bij de ontwikkeling en het handhaven van het botcel fenotype. 1,25-$(OH)_2D_3$ bereikt z'n voornaamste effecten door binding aan z'n receptor, de vitamine D receptor (VDR), die behoort tot de superfamilie van steroid/retinoid/schildklier hormoon receptoren en werkt via de binding aan verschillende vitamine D respons elementen (VDREs) die zich bevinden in de promoter van een aantal genen.

Naast systemische calciotrofe factoren, zoals 1,25-$(OH)_2D_3$, hebben ook verschillende lokaal geproduceerde factoren een effect op het botmetabolisme. Systemische factoren kunnen de produktie van lokale factoren beïnvloeden en daardoor de botvorming en afbraak (resorptie) reguleren. Daarnaast zou de aanwezigheid van lokaal geproduceerde factoren echter ook gevolgen kunnen hebben voor de biologische effecten van systemische factoren. Lokale factoren zouden de werking van systemische factoren kunnen beïnvloeden. De factoren transforming growth factor ß (TGFß), bone morphogenetic protein-2 (BMP-2) en insulin-like growth factor (IGF-I) worden geproduceerd door botcellen en worden opgeslagen in de botmatrix. Het doel van de in dit proefschrift beschreven studie was het vaststellen van de rol van de lokaal geproduceerde factoren TGFß, BMP-2 en IGF-I bij de werking van de systemische factor 1,25-$(OH)_2D_3$ op bot.

Allereerst werd het effect van TGFß op het VDR nivo in botvormende, osteoblast-achtige cellen bepaald. In osteoblasten van verschillende oorsprong verhoogde TGFß op een vergelijkbare manier het VDR nivo (Hoofdstuk 2). Om te bepalen wat het belang is van dit door TGFß verhoogde VDR nivo voor de werking van 1,25-$(OH)_2D_3$ op de expressie van osteocalcine en osteopontine in deze cellen, werden de cellen eerst geïncubeerd met TGFß (preïncubatie leidt tot een verhoogd VDR nivo), en vervolgens werd 1,25-$(OH)_2D_3$ toegevoegd. Osteocalcine en osteopontine zijn niet-collagene botmatrix eiwitten die worden geproduceerd door osteoblasten en waarvan wordt gedacht dat ze een rol spelen bij de botresorptie. Osteocalcine zou betrokken zijn bij het aantrekken en de differentiatie van osteoclasten, de botresorberende cellen. Osteopontine zou betrokken zijn bij de aanhechting van osteoclasten aan het bot. TGFß preïncubatie leidde echter tot een sterke remming van de door 1,25-$(OH)_2D_3$ gestimuleerde osteocalcine en

osteopontine expressie in osteoblast-achtige cellijnen.

In Hoodstuk 3 bestudeerden we het mechanisme waarmee TGFß de door 1,25-$(OH)_2D_3$ verhoogde expressie van osteocalcine en osteopontine remt. ROS 17/2.8 cellen, een osteoblast-achtige cellijn waarin beide genen tot expressie komen, werden getransfecteerd met reporter constructen aangestuurd door de wild-type ratte osteocalcine en muize osteopontine promoters. TGFß verminderde de door 1,25-$(OH)_2D_3$ gestimuleerde transcriptie van zowel het osteocalcine als osteopontine gen. De aanwezigheid van de vitamin D respons elementen (VDREs) bleek noodzakelijk voor de remmende werking van TGFß. Additionele, meer proximaal gelegen basale regulatore elementen bleken echter ook een bijdrage te leveren. Verder werd de bijdrage van *trans*-activerende factoren, die binden aan de osteocalcine en osteopontine VDREs en bestaan uit o.a. de VDR en retinoic X receptor (RXR), aan transcriptionele effecten bestudeerd. Gel mobility shift assays lieten zien dat TGFß de inductie van de binding van heterodimere VDR/RXR complexen aan de VDREs significant remt in met 1,25-$(OH)_2D_3$ behandelde ROS 17/2.8 cellen, en dat dit effect niet tot stand kwam door veranderingen in VDR eiwit nivo. Tevens lieten we zien dat activator protein-1 (AP-1) activiteit, die verhoogd wordt door TGFß, mogelijk een bijdrage levert aan de remmende effecten van TGFß op de basale osteocalcine transcriptie.

Een respons waarvan eerder een koppeling is aangetoond met het VDR nivo is 24-hydroxylase activiteit. Dit enzym is betrokken bij de eerste stap in de C24-oxidatie route van zijketen oxidatie, die wordt gevolgd door afbraak en volledige verwijdering van 1,25-$(OH)_2D_3$ en z'n precursor 25-$(OH)D_3$. Om de betekenis van de regulatie van VDR nivo verder te bestuderen, werd in Hoofdstuk 4 het effect van door TGFß-geïnduceerde verhoging van het VDR nivo op de inductie van 24-hydroxylase activiteit door 1,25-$(OH)_2D_3$ bekeken. Na preïncubatie met TGFß, die het vitamine D receptor nivo verhoogt, bleek ook de door 1,25-$(OH)_2D_3$ geïnduceerde 24-hydroxylase activiteit verhoogd te zijn in twee osteoblast-achtige cellijnen. Door op verschillende manieren het VDR nivo te reguleren, laten we zien dat er een sterke koppeling bestaat tussen VDR nivo en de inductie van 24-hydroxylase door 1,25-$(OH)_2D_3$. Omdat C24-oxidatie onderdeel vormt van de afbraak van 1,25-$(OH)_2D_3$, zou de koppeling van VDR nivo aan de afbraak van 1,25-$(OH)_2D_3$ een belangrijk regulatoor terugkoppelings mechanisme kunnen vormen bij de werking van 1,25-$(OH)_2D_3$ op doelwit weefsel/cel nivo.

In Hoofdstuk 5 werden de effecten van twee andere lokaal geproduceerde

factoren, BMP-2 en IGF-I vergeleken met TGFß. Deze drie groeifactoren bleken verschillende effecten te hebben op de door 1,25-$(OH)_2D_3$ gestimuleerde expressie van osteocalcine en osteopontine en op de binding van VDR/RXR complexen aan de osteocalcine en osteopontine VDREs. Deze resultaten vormen een aanwijzing dat de interactie van groeifactoren met 1,25-$(OH)_2D_3$ groeifactor specifiek is.

In Hoofdstuk 6 werden de effecten van TGFß, BMP-2 en IGF-I op de door 1,25-$(OH)_2D_3$ en bijschildklier hormoon (parathyroid hormoon, PTH) gestimuleerde botresorptie bestudeerd. TGFß bleek de door 1,25-$(OH)_2D_3$ gestimuleerde botresorptie te remmen, maar niet de door PTH gestimuleerde botresorptie. IGF-I remde echter zowel de door PTH als de door 1,25-$(OH)_2D_3$ gestimuleerde botresorptie. BMP-2 had, afhankelijk van de concentratie, een stimulerend effect op de door 1,25-$(OH)_2D_3$ verhoogde botresorptie (10-100 ng/ml), of had (1000 ng/ml) een sterk stimulerend effect op de basale botresorptie, maar geen effect op 1,25-$(OH)_2D_3$ en PTH gestimuleerde botresorptie. Deze gegevens bevestigen de groeifactor specifieke effecten van TGFß, BMP-2 en IGF-I op de werking van 1,25-$(OH)_2D_3$ beschreven in het voorgaande Hoofdstuk. Tevens wordt aangetoond dat de effecten ook hormoon specifiek zijn. Daarnaast hebben we de effecten van lokale en systemische factoren op osteocalcine produktie en collagenase activiteit bestudeerd. Deze factoren zouden betrokken kunnen zijn als mediatoren in de regulatie van de botresorptie. Het was opmerkelijk dat 1,25-$(OH)_2D_3$, in tegenstelling tot het stimulerende effect op botresorptie, een remmend effect had op de osteocalcine eiwit produktie in foetale muize botjes. Dit remmend effect van 1,25-$(OH)_2D_3$ werd ook gezien in de muize osteoblast-achtige cellijn MC3T3. Deze gegevens wijzen op een species specifieke regulatie van osteocalcine door 1,25-$(OH)_2D_3$. 1,25-$(OH)_2D_3$ had geen effect op de collagenase activiteit.

Analyse van de verdeling van lokaal geproduceerde factoren in de botmatrix toonde aan dat in volwassen humaan bot de aanwezigheid van TGFß type 2, TGFß type 3, BMP-2 en IGF-I beperkt is tot specifieke plaatsen en dat ze niet diffuus verspreid aanwezig zijn. Deze observatie kan erop duiden dat lokaal geproduceerde factoren de respons op systemische factoren op specifieke plaatsen in bot bepalen. De hoeveelheid en het type groeifactor dat op specifieke plaatsen in de botmatrix aanwezig is, zou tot een verlaagde of juist een verhoogde botresorptie in respons op 1,25-$(OH)_2D_3$ en/of PTH kunnen leiden.

Variatie in de sequentie van de osteocalcine en osteopontine VDREs kan leiden tot herkenning van deze elementen door verschillende hormoon receptor homo- of

heterodimeren. In Hoofdstuk 7 tonen we aan dat de door 1,25-$(OH)_2D_3$ geïnduceerde complexen die aan de osteocalcine en osteopontine VDREs binden, verschillende complexen zijn, waarvan de VDR en RXR type α deel uit maken. De 2 middelste nucleotiden in het proximale half element van de osteocalcine VDRE (5'-AGGACA) bleken bepalend te zijn voor de verschillen in immunoreactiviteit van een VDR antilichaam. De resultaten in Hoofdstuk 7 wijzen op polarisatie van de binding van het VDR/RXRα complex aan de osteocalcine en osteopontine VDREs, waarbij de proximaal gelegen half-elementen van de VDREs worden herkend door de VDR. Tevens zijn er aanwijzingen dat het DNA bindend domein van de VDR verschillende eiwit conformaties aan neemt bij binding aan de verschillende VDREs. Dit zou fysiologische gevolgen kunnen hebben voor de regulatie van deze twee genen gedurende de instandhouding van de bothomeostase.

De in dit proefschrift beschreven studie toont aan dat de lokaal geproduceerde factoren TGFß, BMP-2 en IGF-I belangrijke effecten hebben op de werking van de systemische factor 1,25-$(OH)_2D_3$ en niet alleen dienen als mediatoren waardoor het effect van 1,25-$(OH)_2D_3$ op bot tot stand komt. Deze interacties tussen lokale en systemische factoren zouden in het algemeen een belangrijke rol kunnen spelen in de effecten van hormonen op hun doelwit weefsel.

ACKNOWLEDGMENTS

None of these results would have been achieved without the help of many others. First of all, I would like to thank Prof. Dr. J.C. Birkenhäger for his unflagging interest in my work, for the quick reading of my manuscripts and for stimulating discussions. Secondly, I thank Dr. Hans van Leeuwen and Dr. Huib Pols for the enormous amount of time and enthusiasm that they invested in teaching me "science": planning of experiments, discussing data, writing abstracts and manuscripts, making posters and preparing talks. Furthermore, I am very grateful for the opportunity they offered me to perform part of the experiments at the Department of Cell Biology at the University of Massachusetts Medical Center in Worcester (Massachusetts, USA), where I learned several techniques.

Furthermore, I got a lot of practical help from the people in the "vitamin D lab". First of all, I would like to thank Wendy Geertsma-Kleinekoort, who performed the RNA experiments, and together with Gert-Jan van den Bemd performed many bone resorption studies. Gert-Jan was also of great help with the 24-hydroxylase studies, for which he performed the HPLC runs. Cok Buurman was very helpful by measuring osteocalcin in a lot of medium samples. I thank all people of the "vitamin D lab", including also Dr. Trudy Vink-van Wijngaarden, Edgar Colin, Dr. André Uitterlinden and QuiJu Huang for their practical help, usefull discussions and an unforgettably enjoyable time. Furthermore, I would like to thank all other people from the Department of Internal Medicine III for their help and a lot of (volleyball) fun.

I thank Pieter Derckx from the Department of Pathology for his great help with the immunohistochemistry, Dr. Clemens Lowik from the Department of Endocrinology and Metabolism of the University of Leiden for providing us with samples of mouse osteoblast-like cells for osteocalcin measurements and Dr. V. Everts from the Department of Cell Biology and Histology of the University of Amsterdam for measurements of collagenase activity in the medium of fetal mouse long bone cultures.

From the Department of Cell Biology of the University of Massachusetts Medical Center I would like to thank Dr. Gary Stein, Dr. Jane Lian and Dr. Janet Stein for the great opportunity to work in their lab and for the many stimulating discussions on experiments and manuscripts. This period was productive as well as very enjoyable. Many people of the Department of Cell Biology were of great help. I thank Jack Green and Liz Buffone for cell culture, Rosa Mastrototaro for technical assistance, Casey Caparelli and Danielle Lindenmuth for osteocalcin assays, Chaitali Banerjee, Rajesh Desai, Dr. Vicki Shalhoub and Dr. Baruch Frenkel for practical advise. Furthermore, I would like

to thank Dr. André van Wijnen, who was not only of great scientific help with practical advise and stimulating discussions, but who was also of great help with arranging my social life in Worcester. I thank all people of the Department of Cell Biology for practical help, useful discussions, basketball fun and a great time.

Last but not least, I would like to thank my family and friends, especially my mother and Marcel, for their interest and encouragements.

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