Vitamin D
Action and regulation in bone cells

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VITAMIN D

ACTION AND REGULATION IN BONE CELLS

VITAMIN D

EFFECTEN EN REGULATIE IN BOTCELLEN

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. A.H.G. RINNOOY KAN
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 22 JUNI 1988 TE 13.45 UUR

DOOR

HUIBERT ADRIAAN PIETER POLS

GEBOREN TE DORDRECHT

PROMOTIECOMMISSIE:

PROMOTOREN:	PROF. DR. J.C. BIRKENHÄGER PROF. DR. IR. T.J. VISSER
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The studies reported in this thesis were carried out at the Department of Internal Medicine III and of Clinical Endocrinology (head Prof. Dr. J.C. Birkenhäger), Erasmus University Medical School, The Netherlands.
The investigations were supported by a grant from the Dutch Kidney Foundation (No C83.401).
The support from LEO Pharmaceuticals BV for the publication of this thesis is gratefully acknowledged.

Aan mijn ouders,

Voor Lientje, David en Thomas

Contents

List of abbreviations

BSA	bovine serum albumin
BSS	buffered salt solution
cAMP	cyclic adenosine 3',5'-monophosphate
CaBP	Calcium binding protein
DBP	Vitamin D binding protein
FCS	fetal calf serum
Kd	apparent affinity
MEM	Eagle's minimum essential medium
mRNA	messenger ribonucleic acid
Nmax	maximum number of binding sites
OB	osteoblast-like
PTH	parathyroid hormone
SCA	specific cellular accumulation
1,25-$(OH)_2D_3$	1,25-dihydroxyvitamin D_3
1,24,25-$(OH)_3D_3$	1,24,25-trihydroxyvitamin D_3
24-oxo-1,25-$(OH)_2D_3$	24-oxo-1,25-dihydroxyvitamin D_3
24-oxo-1,23,25-$(OH)_3D_3$	24-oxo-1,23,25-trihydroxyvitamin D_3
Vmax	maximum velocity

Section I

Vitamin D

Action and regulation in bone cells

CHAPTER 1

The metabolism, mechanism of action and biological role of vitamin D

1.0 Historical background

Studies of the function and endocrinology of vitamin D_3 over the last 80 years have elucidated its role as an important regulator of calcium and phosphate homeostasis. During this period our understanding of the significance of vitamin D has greatly increased. First, the identification by Sir Edward Mallanby of the antirachitic factor (later termed vitamin D) and its importance in controlling blood calcium and phosphate levels was a major insight in basic physiology and had important therapeutic implications (Mallanby, 1919; Steenbock et al, 1924; Hess et al, 1924; Askew et al, 1931). The next perhaps most important historical development has been the demonstration that vitamin D must be metabolically altered to the hormone 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), before it can exert its well-known biological activities (Fraser and Kodicek, 1970). Subsequently, investigators characterized a specific receptor for 1,25-$(OH)_2D_3$ in one of the classical target tissues, the intestine (Brumbaugh et al, 1973). Current data show that a wide variety of tissues and cells both have receptors for the sterol and show biological responses to the hormone (Haussler, 1986). Often, these other target tissues do not take part in the regulation of serum calcium homeostasis, but the presence of vitamin D receptors in these cells appears to be related to their state of differentiation/proliferation.

1.1 Metabolism of vitamin D

1.1.1 Sources of vitamin D

There are two sources from which vitamin D_3 (cholecalciferol) is normally provided to man: it is produced in the skin and the intact vitamin is also absorbed from the diet. Vitamin D_2 (ergocalciferol) is derived from plant sterols and used as a therapeutic agent or for the fortification of foods.

The sequence of events leading to the formation of vitamin D_3 in the skin is clearly established. During exposure to sunlight, the epidermal cutaneous reservoir of 7-dehydrocholesterol, which is largely present in the Malphigii layer, is photochemically converted to provitamin D_3, which then isomerizes to vitamin D_3 over an interval of two to three days. Vitamin D_3 is removed from the skin by vitamin D binding protein, which has a much higher affinity for vitamin D_3 than for the pro-

vitamin D_3. This results in a low vitamin D_3 concentration in the skin, allowing the thermal isomerization of provitamin D_3 to vitamin D_3 to proceed (for review DeLuca, 1984; Henry and Norman, 1984; Kumar 1986).

1.1.2 The formation of 25-hydroxyvitamin D_3 in the liver

Predominantly in the liver but not exclusively so, vitamin D_3 is hydroxylated on carbon 25 to 25-hydroxyvitamin D_3 (25-(OH)D_3). Some 25-hydroxylation can also take place in the intestine and kidney, although the amounts produced are small inasmuch as hepatectomized animals produce little 25-(OH)D_3 in vivo (Tucker et al, 1973; Bhattacharyya et al, 1974; Olsen et al, 1976).

It is not known which of the cells in the liver carry out the hydroxylation, although of course the hepatic cell is the best candidate. The 25-hydroxylase is present both in liver microsomes as well as in liver mitochondria. The microsomal enzyme requires NADPH and a soluble cytoplasmic factor for its activity. It is a cytochrome P-450 enzyme system that has recently been characterized (Yoon and DeLuca, 1980; Dahlback and Wikvall, 1987). Previous data suggested that the microsomal enzyme has a lower Michaelis constant (K_m, 10^{-8} M) than the mitochondrial enzyme, which has a K_m of 10^{-6} M (Bjorkhem et al, 1978 and 1980). The concept that the microsomal 25-hydroxylation of vitamin D_3 is the most important system under physiological conditions was recently questioned by Dahlback and Wikvall (1987). These investigators reported a lack of correlation between the microsomal cytochrome P-450-dependent 25-hydroxylation of vitamin D_3 and the serum 25-(OH)D_3 level of rats. On the other hand, the mitochondrial 25-hydroxylase activity and the serum 25-(OH)D_3 levels showed a good correlation. Furthermore, Saarem et al (1984) showed that human liver only contained one detectable vitamin D_3 25-hydroxylase and that this system was located at the inner mitochondrial membrane. Therefore, these data suggest that the mitochondrial cytochrome P-450 might play a more important role in 25-hydroxylation of vitamin D_3 than is currently thought.

The administration of 1,25-$(OH)_2D_3$ has been shown to lower the concentration of 25-(OH)D_3 in plasma in vivo (Bell et al, 1984). Recently, Clements et al (1987) showed in rats that the half-life of 25-(OH)D_3 in the liver was reduced by calcium deprivation. It was suggested that 1,25-$(OH)_2D_3$, produced in response to secondary hyperparathyroidism, mediates this effect. Conversely, phosphate deprivation (which raises the 1,25-$(OH)_2D_3$ concentration in plasma) does not alter 25-(OH)D_3 concentration in blood (Van den Berg et al, 1980). Therefore, the physiological significance of 1,25-$(OH)_2D_3$ in the regulation of 25-(OH)D_3 levels is not clear and needs further study.

1.1.3 The formation of 1,25-dihydroxyvitamin D_3

The hormonal form of vitamin D_3, 1,25-$(OH)_2D_3$, is formed in the mitochondria of

the proximal tubules of the nephron, as originally found by Fraser and Kodicek (1970). The 25-(OH)D_3-1α-hydroxylase is a cytochrome P-450 mixed function oxidase that utilizes molecular oxygen and NADPH (for detailed description of this enzyme, see DeLuca, 1984). There are several factors that may regulate the activity of the 1α-hydroxylase. Some of these factors have this function only in certain species and under certain conditions. A summary of various factors influencing 1,25-$(OH)_2D_3$ synthesis is given in Table I. The major factors regulating the activity of the 1α-hydroxylase are parathyroid hormone, the concentration of serum phosphate, 1,25-$(OH)_2D_3$ itself and perhaps serum calcium directly. The further relationships among calcium controlling hormones, other factors and the vitamin D endocrine system have already been extensively reviewed by others (Henry and Norman, 1984; Audran and Kumar, 1985; Kumar, 1986).

Although there is compelling evidence that 1α-hydroxylase is mainly localized in the kidney, the original hypothesis that 1,25-$(OH)_2D_3$ is synthesized there exclusively is not tenable. At present there is no doubt that in a pregnant mammal 1,25$(OH)_2D_3$ is in fact produced in placental tissue (Weissman et al, 1978; Gray et al, 1979; Tanaka et al, 1979). Furthermore, hypercalcemia in patients with granulomatous diseases (i.e. sarcoidosis, tuberculosis, berylliosis and coccidiomycosis) and certain malignancies, is explained by extrarenal 1,25-$(OH)_2D_3$ production (for review Mason, 1985). Recently, lymph-node homogenates from patients with sarcoidosis have been shown to be able to synthesize the active form of vitamin D_3 (Mason et al, 1984). At the cellular level the macrophages represent likely candidates for this extrarenal 1,25-$(OH)_2D_3$ synthesis (Adams et al, 1983; Koeffler et al, 1985).

The significance of 1α-hydroxylase activity in bone will be discussed in chapter 3.

1.1.4 Formation of other dihydroxyvitamin D_3 metabolites

Many studies showed that other pathways of vitamin D_3 metabolism exist. All these pathways utilize 25-(OH)D_3 as substrate. As shown in Fig. 1, 25-(OH)D_3 can also be converted to 24,25-$(OH)_2D_3$, 25,26-$(OH)_2D_3$ and 23,25-$(OH)_2D_3$ (for review DeLuca, 1984; Henry and Norman, 1984).

24-hydroxylation: Besides the 1α-hydroxylation, this pathway has received the greatest attention. 24,25-$(OH)_2D_3$ is a major metabolite found in human plasma (Gray et al, 1974). The enzyme 24-hydroxylase is found in a variety of tissues and cells like kidney (Knutson et al, 1974), intestine (Kumar et al, 1978), cartilage (Garabedian, 1978), osteoblasts (Pols et al, 1984) and fibroblasts (Griffin et al, 1983). The hydroxylase is a mitochondrial cytochrome P-450 enzyme system. It requires NADPH and is likely to be a three component mixed function oxidase analogous to the 1α-hydroxylase (Knutson et al, 1974). Data with respect to the relative Km values of the 24-hydroxylase in the different tissues are not available. Only for the kidney a Km for 25-(OH)D_3 of around 10^{-8} M has been reported (DeLuca, 1984). In vitamin

Table I: Factors altering serum 1,25-$(OH)_2D_3$ levels or 25-$(OH)D_3$-1α-hydroxylase activity.

		Effect on 1,25-$(OH)_2D_3$ level or 1α-hydroxylase activity	
Factor	Level or activity change	Animal	Human
Parathyroid hormone	increase	+	+
	decrease	−	−
Serum phosphate	increase	+	+
	decrease	−	−
1,25-$(OH)_2D_3$	increase	−	?
	decrease	+	?
Calcium	increase	?	?
	decrease	+	+
Calcitonin	increase	+,0,−	+
	decrease	?	?
Hydrogen ion	increase	−	0
	decrease	?	?
Estrogen	increase	+,0	+
	decrease	?	?
Prolactin	increase	+	0
	decrease	?	?
Growth hormons	increase	+	+,0,−
	decrease	?	?
Glucocorticoids	increase	−	+,0,−
	decrease	?	?
Thyroid hormone	increase	?	−*
	decrease	?	+*
Pregnancy	−	+	+

(+) stimulation or increase; (−) suppression or decrease; (0) no effect
(*) effects may be caused by changes in calcium, phosphate or parathyroid hormone. Modified from Kumar (1984) and Henry and Norman (1984).

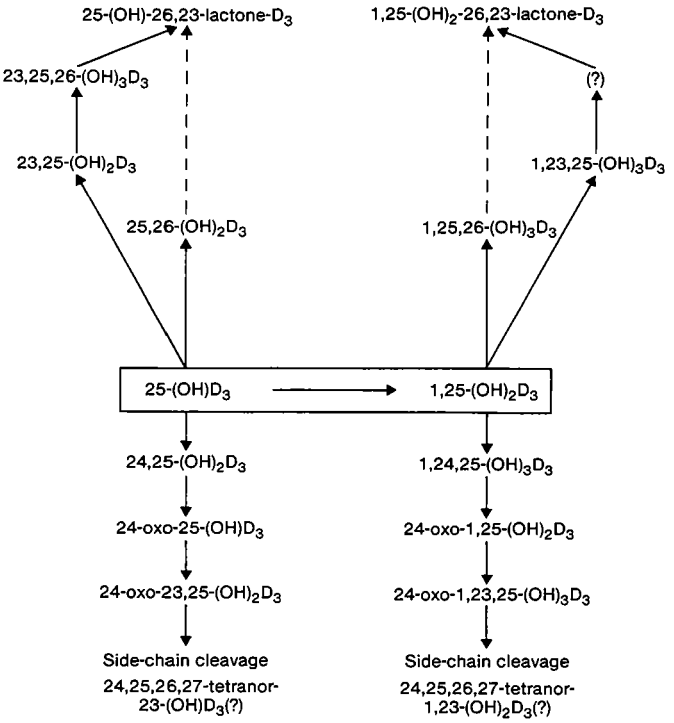

Fig. 1: Summary of vitamin D_3 metabolism. Metabolites are organized into logical pathways. The lower part represents the C23- and C24-oxidation pathway of 25-(OH)D_3 and 1,25-(OH)$_2D_3$.

D_3 deficiency 24-hydroxylase activity is undetectable, while enzyme activity is induced and regulated by 1,25-$(OH)_2D_3$ (Tanaka et al, 1975).

Recently, Jones et al (1983) showed that 24,25-$(OH)_2D_3$ is rapidly further converted to 24-oxo-25-(OH)D_3, followed by C23-hydroxylation and likely by side-chain cleavage to yield a C23 carboxyl metabolite. That the C24-oxidation of 25-(OH)D_3 is not specific for this metabolite will be discussed below (1.1.5).

26-hydroxylation: This represents a minor pathway in the metabolism of 25-(OH)D_3. The enzyme 26-hydroxylase is found in the kidney, but little is known about its enzymology (Tanaka et al, 1978). DeLuca (1984) considers 26-hydroxylation as an interesting biological curiosity.

23-hydroxylation: 23,25-$(OH)_2D_3$ is formed in the kidney (Tanaka et al, 1980), but also extrarenally (Napoli et al, 1982). It is an intermediate in the biosynthesis of 25-(OH)D_3-26,23-lactone (Tanaka et al, 1980). The lactone is only found under conditions of vitamin D_3 intoxication.

Finally, all the above-mentioned dihydroxyvitamin D_3 derivatives can be converted to their respective 1α-hydroxylated metabolites in vitro, but these hydroxylations do not seem of significance in vivo (DeLuca, 1984).

1.1.5 The catabolism of 1,25-dihydroxyvitamin D_3

Derangements in 1,25-$(OH)_2D_3$ tissue or plasma levels could result from alterations in its synthesis rate, degradation rate or both. Therefore, it is important to investigate the further metabolic pathways of the sterol and their physiological control. The metabolic pathways involved in the degradation of 1,25-$(OH)_2D_3$ are as follows (for review Kumar, 1984):

- Side-chain oxidation (1.1.5.1)
- C24-oxidation (1.1.5.2)
- Formation of 1,25-$(OH)_2D_3$-26,23-lactone (1.1.5.3)
- Formation of 1,25,26-$(OH)_3D_3$ (1.1.5.4)
- Biliary excretion of polar metabolites (1.1.5.5)

1.1.5.1 Side-chain oxidation:

Experiments in rats showed that after intravenous administration of [26,27-^{14}C]1,25-$(OH)_2D_3$ around 20-25% was converted to $^{14}CO_2$ within 24 h (Harnden et al, 1976; Kumar et al, 1977). This process continued unchanged in anephric animals, while removal of the entire small intestine and large bowel was associated with a substantial decline in the rate of $^{14}CO_2$ production in the expired air. Bacterial metabolism did not have any influence, because germ-free rats produced as much $^{14}CO_2$ as normal rats (Kumar et al, 1977). These results indicate that the intestine and/or the liver are involved in the side-chain oxidation of 1,25-$(OH)_2D_3$. No changes in $^{14}CO_2$ production were found in rats depleted of or repleted with vitamin D_3, calcium or phosphate. Also pretreatment with 1,25$(OH)_2D_3$ did not influence this overall process. Similar experiments with [26,27-^{14}C]25-$(OH)D_3$ showed that removal of the kidneys abolished $^{14}CO_2$ production, thereby suggesting that the whole degradation of 25-$(OH)D_3$ is dependent upon the presence of 1,25-$(OH)_2D_3$ (Kumar et al, 1977).

Calcitroic acid (1α-hydroxy-23-carboxy-24,25,26,27-tetranor vitamin D_3), a side-chain cleavage metabolite of 1,25-$(OH)_2D_3$ was found in the liver of rats dosed with [3-^{3}H]1,25-$(OH)_2D_3$. It probably represents an excretory or degradative product of 1,25-$(OH)_2D_3$ (Esvelt and DeLuca, 1981).

1.1.5.2 C24-oxidation:

Using rat intestinal mucosa homogenates, Napoli et al (1983) were able to demonstrate the formation of several metabolites of 1,25-$(OH)_2D_3$ which can be logically placed in a single metabolic pathway:

1,25-$(OH)_2D_3$ → 1,24,25-$(OH)_3D_3$ → 24-oxo-1,25-$(OH)_2D_3$ → 24-oxo-1,23,25-$(OH)_3D_3$ → side-chain cleavage (calcitroic acid?) The same investigators demonstrated that 1,24,25-$(OH)_3D_3$ and 24-oxo-1,25-$(OH)_2D_3$ are present in vivo in plasma and intestinal tissue of rats given regular vitamin D-containing diets and in enhanced amounts in the intestine of rats dosed chronically with 1,25-$(OH)_2D_3$. The finding that 24-hydroxylation of 1,25-$(OH)_2D_3$ is not substantially impaired in anephric animals provides further evidence for an important extrarenal C24-oxidation (Kumar et al, 1984). Recently, it has been shown that the C24-oxidation pathway also exists in other tissues (Mayer et al, 1983; Chandler et al, 1984; Eisman et al, 1984).

The metabolic pathways of 25-$(OH)D_3$ and 1,25$(OH)_2D_3$ initiated by 24-hydroxylation appear to be analogous (Fig. 1). In this respect it is important to note that 1,25-$(OH)_2D_3$ is the main inducer and regulator of 24-hydroxylase activity in a wide variety of target tissues (section 1.1.4). In other words, 1,25-$(OH)_2D_3$ induces its own metabolism by initiating 24-hydroxylase activity. For more detailed information about the precise role of C24-oxidation the reader is referred to the chapters 3 and 4.

1.1.5.3 Formation of 1,25-$(OH)_2D_3$-26,23-lactone:
It is not known whether this metabolite may be found under normal circumstances in vivo. Like the corresponding lactone of 25-$(OH)D_3$, 1,25-$(OH)_2D_3$-26,23-lactone is only found in animals dosed with large amounts of vitamin D_3 (Ohnuma et al, 1980). Consequently, regulation of this pathway is still uncertain.

1.1.5.4 Formation of 1,25,26-$(OH)_3D_3$:
Tanaka et al (1981) isolated this metabolite of 1,25-$(OH)_2D_3$ from kidney homogenates of vitamin D-supplemented chicks. Although this observation suggests that it is possible for 1,25,26-$(OH)_3D_3$ to be generated from 1,25-$(OH)_2D_3$ in vivo, proof is currently not available.

1.1.5.5 Biliary excretion of polar metabolites:
Side-chain oxidation and 24-hydroxylation of 1,25-$(OH)_2D_3$ account for approximately 35-40% of 1,25-$(OH)_2D_3$ metabolism (Kumar, 1984). Clearly other routes must exist for the metabolism of the sterol. Kumar and coworkers reasoned that the large amount of radioactivity found in the feces after intravenous administration of radiolabelled 1,25-$(OH)_2D_3$ to rats could be derived from biliary excretory products. Indeed they found a large amount of conjugated polar 1,25-$(OH)_2D_3$ metabolites in the bile (Kumar et al, 1980). These metabolites are in the form of glucuronides and other charged compounds that may be sulfates of 1,25-$(OH)_2D_3$ or its metabolites (Kumar, 1984). Also neutral substances are found, which probably represent

glycosides of the hormone. The metabolites of 1,25-$(OH)_2D_3$ undergo an enterohepatic recirculation that may be perturbed in certain pathologic states (Kumar et al, 1983).

Finally, the biliary excretion of 1,25-$(OH)_2D_3$ is an unsaturable process. Also dietary changes of calcium and phosphate do not influence the amount of polar metabolites of 1,25-$(OH)_2D_3$ found in the bile (Kumar, 1984).

1.1.6 Summary

The synthesis of 1,25-$(OH)_2D_3$ is controlled by circulating amounts of parathyroid hormone, serum or extracellular phosphate levels, circulating levels of 1,25-$(OH)_2D_3$ and perhaps serum calcium directly. Many of the other factors noted have effects in vitro only or effects that are observed in one or a few species only. Under certain conditions 1,25-$(OH)_2D_3$ can also be synthesized extrarenally. The precise role and control of this phenomenon is unknown.

The metabolism of 1,25-$(OH)_2D_3$ to other metabolites appears to be mainly a degradative or excretory process. Important processes such as side-chain oxidation and biliary excretion are not regulated by calcium, phosphate and 1,25-$(OH)_2D_3$. Only the C24-oxidation pathway is induced by 1,25-$(OH)_2D_3$, which could serve as a defence against excesses of 1,25-$(OH)_2D_3$ in various tissues by conversion of the hormone to less active or inactive metabolites (see Chapter 4).

1.2 Mechanism of action of vitamin D

1.2.1 General model of action

For the understanding of the mechanism of action of vitamin D, the characterization of its receptor in target tissues was of major importance. The vitamin D receptor was first revealed in 1968 as a chromosomal protein in intestinal mucosa nuclei that specifically bound an active metabolite of the vitamin (Haussler et al, 1968). This metabolite was identified two years later as 1,25-$(OH)_2D_3$ (Fraser and Kodicek, 1970).

Biochemical studies of the so-called '1,25-$(OH)_2D_3$ receptor' indicate that the mode of action of vitamin D is similar to that of steroid hormones (for review Haussler, 1986). The present working hypothesis (Fig. 2) is that the 1,25-$(OH)_2D_3$ receptor is a loosely associated chromosomal protein with increased affinity for DNA upon hormone binding. It is probable that a fraction of unoccupied receptors for 1,25-$(OH)_2D_3$ exists in the cytoplasm in equilibrium with the majority of unoccupied receptors in the nucleus (Hunziker et el, 1980; Walters et al, 1980; Pike, 1982; Pike and Haussler, 1983). Whatever the precise subcellular distribution of the receptor may be, it is more important that the occupied receptor apparently is capable to regulate gene expression. The mRNA's formed as a result of the interaction of the

hormone-receptor complex with the genome are translated on the ribosomes into new proteins that mediate the biological response of the cell to the hormone (see section 1.3).

1.2.2 The 1,25-dihydroxyvitamin D_3 receptor

The 1,25-$(OH)_2D_3$ receptor is a DNA-binding protein with a molecular weight of 50,000 – 60,000 daltons. It binds 1,25-$(OH)_2D_3$ with very high affinity (Kd:

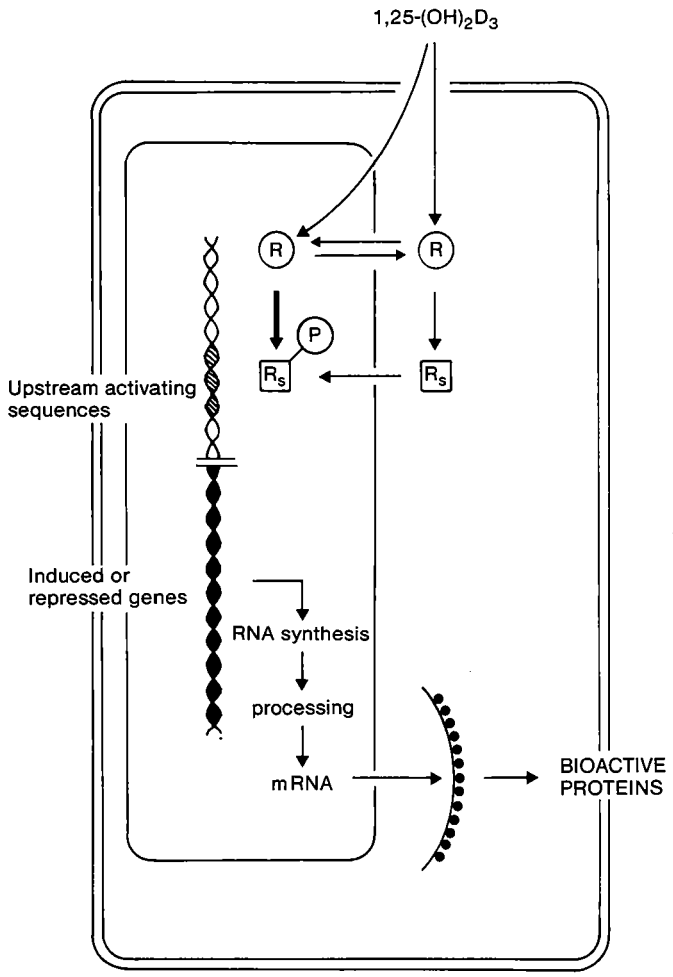

Fig. 2: Model of 1,25-$(OH)_2D_3$ action.
An R in a circle represents the unoccupied receptor and an R_S designates the occupied (phosphorylated) receptor.

10^{-10}-10^{-11} M) and selectively over other vitamin D metabolites (Napoli and Horst, 1984). The isolated receptor possesses distinct domains for 1,25-$(OH)_2D_3$ binding and for interaction with DNA (Allegretto et al, 1987). Recently, two receptor-specific monoclonal antibodies were used to isolate and clone the complementary DNA (cDNA) encoding the 1,25-$(OH)_2D_3$ receptor. The amino acid sequence deduced from this cDNA revealed a highly conserved cysteine-rich region that displayed homology with a domain characteristic of other steroid receptors (McDonnell et al, 1987).

That the 1,25-$(OH)_2D_3$ receptor also undergoes qualitative modification, through phosphorylation of the receptor protein upon hormone binding, was observed in cultures of mouse 3T6 fibroblasts by Pike et al (1985). Recently, a similar phosphorylation of the nuclear progesterone receptor in uterine slices was reported (Logeat et al, 1985). In vitro translation of 3T6 cell mRNA, followed by immunoprecipitation of receptor, showed that this phenomenon is a posttranslational process (Mangelsdorf et al, 1987). Some investigators suggest that the phosphorylated receptor could be the form of the receptor that binds most avidly to upstream regulatory regions of the vitamin D-controlled genes (Haussler, 1986). At present, however, definitive proof is not available.

The 1,25-$(OH)_2D_3$ receptor has been detected in mammals, birds and amphibians, where it is prominent in such tissues as skin, gut and kidney. This indicates that the receptor is an evolutionary ancient molecule. The cross-reactivity of monoclonal antibodies to chick (Pike et al, 1982, 1983) and porcine 1,25-$(OH)_2D_3$ receptors (Dame et al, 1985) supports this suggestion.

In conclusion, based upon its molecular size, amino acid sequence, DNA-binding properties, subcellular location and capacity to induce the synthesis of new proteins, the 1,25-$(OH)_2D_3$ receptor bears striking resemblance to the estrogen receptor and appears to belong to 'the super-family' of steroid hormone receptors.

1.2.3 Is 1,25-dihydroxyvitamin D_3 the only biologically active vitamin D metabolite?

There are now many lines of evidence indicating that 1,25-$(OH)_2D_3$, through its specific receptor mediates most, if not all, biological functions of vitamin D. Some investigators, however, claimed a biological role for other 25-$(OH)D_3$ metabolites. In this respect 24,25-$(OH)_2D_3$ received the greatest attention (for reviews Norman et al, 1980, 1982; Henry and Norman, 1984).

The evidence against a possible unique action of 24,25-$(OH)_2D_3$ was recently reviewed by Brommage and DeLuca (1985). These investigators performed extensive experiments with 24,24-difluoro-25-$(OH)D_3$ in which 24-hydroxylation is (presumably) blocked by the presence of the fluorine atoms. This compound exerts the same effects as 25-$(OH)D_3$ and more specifically 1,25-$(OH)_2D_3$ in several classical bioassays of vitamin D action. These results suggest that effects of 24,25-$(OH)_2D_3$,

mostly observed in a vitamin D-deficient state, occur after 1α-hydroxylation of this compound to 1,24,25-$(OH)_3D_3$, and subsequent activation of the 1,25-$(OH)_2D_3$ receptor. It is well known that vitamin D_3 metabolites have a greater affinity for the 1,25-$(OH)_2D_3$ receptor after 1α-hydroxylation (Stern, 1981). Furthermore, it is important to recognize that in contrast to the relatively high affinity of the receptor for 1,25-$(OH)_2D_3$, compared to other vitamin D metabolites, the serum vitamin D-binding protein (DBP) shows a relatively low affinity for the hormone. Thus DBP binds 24,25-$(OH)_2D_3$ more avidly than 1,25-$(OH)_2D_3$, which allows the intracellular receptor selectively to concentrate and retain 1,25-$(OH)_2D_3$ in vitamin D target cells (Manolagas and Deftos, 1981).

On the other hand, evidence in support of a biologically significant role of 24,25-$(OH)_2D_3$ is provided by observations suggesting the existence of a receptor-like protein for 24,25-$(OH)_2D_3$ in cartilage of chicks (Merke et al, 1981; Somjen et al, 1982). However, the key requirement that such a receptor must have a high affinity for 24,25-$(OH)_2D_3$ compared to other vitamin D_3 metabolites is still not answered. Furthermore, it is also questionable whether observations in chicks are valid for other species.

In summary, the majority of data suggest that 1,25-$(OH)_2D_3$ is the biologically active vitamin D metabolite. The question then left to be answered is the function of the 24-hydroxylase in the vitamin D endocrine system. Experiments to test the hypothesis that this enzyme serves to regulate the 1,25-$(OH)_2D_3$ concentration in its target tissues are discussed elsewhere in this thesis (Chapters 3 and 4).

1.3 Biological role of 1,25-dihydroxyvitamin D_3

1.3.1 Introduction

It is well established that the active metabolite of vitamin D_3, 1,25-$(OH)_2D_3$, is of major physiological importance in controlling calcium homeostasis. This 'classical' picture has become confused of late by extensive reports that a wide variety of tissues and cells both have receptors for 1,25-$(OH)_2D_3$ and respond to the sterol by changes in their biological activity (Table II). These other targets are, in most cases, not obviously part of the mechanisms that regulate serum calcium levels ('non-classical targets'). In general, two biological responses to 1,25-$(OH)_2D_3$ have been linked with the presence of receptors: firstly, decreased proliferation and enhanced differentiation, and secondly the presence of calcium-binding protein (review Braidman and Anderson, 1985).

In the following sections the effects of 1,25-$(OH)_2D_3$ in the so-called 'classical' and 'non-classical tissues' will be reviewed.

Table II: Relevant tissues and cells that possess 1,25-$(OH)_2D_3$ receptors.

Category	Tissue	Specific cell types
Calcium homeostasis	Intestine	Absorptive epithelial
	Kidney	Distal epithelial
	Bone	Osteoblasts
	Parathyroid	Chief cells
Immune system	Blood and bone marrow	Activated lymphocytes, Monocytes
Endocrine	Pancreas	B-cell
	Pituitary	Somatomammotrophic cells
	Ovary	?
	Testis	Seminiferous/Sertolicells
	Thyroid	C-cells
Reproductive	Breast	Epithelial cells
		Breast cancer cells
	Placenta	?
	Uterus	?
Other	Skin	Fibroblasts
	Muscle	Myoblasts
	Brain	Certain neurons
		Hippocampus
	Colon	Colon carcinoma cells

Modified from Braidman and Anderson (1985) and Haussler (1986).

1.3.2 Calcium homeostasis

The mechanism by which vitamin D_3 controls calcium homeostasis has been extensively reviewed by others (Haussler and McCaine, 1977; DeLuca, 1984; Audran and Kumar, 1985; Kumar, 1986). Therefore, only the main issues and new developments will be discussed.

A decrease in plasma calcium is rapidly sensed by the parathyroid glands, resulting in an increased secretion of parathyroid hormone (PTH). In addition to the direct effects of PTH on calcium mobilization from bone and on the fractional calcium excretion in the kidney, the hormone stimulates the 25-$(OH)D_3$-1α-hydroxylase, which in turn enhances the synthesis of 1,25-$(OH)_2D_3$. The latter substance then plays a crucial role in the complex regulation of calcium homeostasis.

1.3.2.1 The effect of 1,25-dihydroxyvitamin D_3 on intestinal calcium transport.

The exact sequence of biochemical changes in the intestinal cells in response to

1,25-$(OH)_2D_3$ is uncertain, although most investigators currently agree that the effect on calcium movements across the enterocyte are dependent upon de novo protein synthesis and are 1,25-$(OH)_2D_3$ receptor-mediated (Corradino, 1973; Franceschi and DeLuca, 1981). A number of proteins (i.e. calcium-binding protein (CaBP), intestinal membrane calcium-binding protein) and enzymes (e.g. alkaline phosphatase) have been shown to increase in concentration, turnover or activity after the administration of vitamin D or its active metabolite to vitamin D-deficient animals (for review Wasserman et al, 1984). The most prominent among these is certainly CaBP. This protein was first detected in chick intestine (Wasserman and Taylor, 1966) and later shown to be a unique gene product of 1,25-$(OH)_2D_3$ action (Desplan et al, 1983). Although it is proposed that CaBP is concerned with mechanisms of active calcium transport it does not seem to act as 'a buffer' for intracellular calcium (Wasserman et al, 1984).

There is now considerable evidence that 1,25-$(OH)_2D_3$ has also multiple effects both at the luminal membrane and the basolateral membrane of the enterocyte. For instance calcium uptake at the luminal membrane seems not to be dependent upon the synthesis of new protein. Rasmussen et al (1982) have suggested that 1,25-$(OH)_2D_3$ induces an alteration in the phospholipid character of the brush border, resulting in a change in membrane fluidity. This, in turn, might increase the calcium permeability of the membrane. However, changes in membrane fluidity have not been found by others (Wasserman et al, 1984). Also, the role of the phosphorylation of certain brush border proteins in the regulation of calcium uptake is not well understood (Wasserman and Brindak, 1979; De Jonge, 1981; Braun, 1984).

Ghijsen and Van Os (1982) have noted an increase in the activity of a calcium-dependent ATPase at the basolateral membrane following administration of 1,25-$(OH)_2D_3$. This process may be important in the movement of calcium out of the cell and into the extracellular fluid.

Summarizing the effects of 1,25-$(OH)_2D_3$ on intestinal calcium transport, it seems that the luminal membrane calcium uptake may not be protein-dependent, while the movement of the cation across the cell does depend upon a 1,25-$(OH)_2D_3$ receptor-mediated synthesis of proteins. Contraluminal enzymes such as calcium ATPase are most probably involved in the extrusion of calcium out of the cell. However, there is no agreement on the time sequence of the 1,25-$(OH)_2D_3$-induced events nor on the rate-limiting step in transepithelial calcium transport. Finally, 1,25-$(OH)_2D_3$ also stimulates intestinal phosphate transport (Harrison, 1961; Chen et al, 1974). The molecular mechanism of this proces is not known.

1.3.2.2 The effect of 1,25-dihydroxyvitamin D_3 on calcium mobilization from bone. The precise role of bone in systemic control of the extracellular level of calcium by

$1,25\text{-}(OH)_2D_3$ is uncertain and it is doubtful whether it is as crucial to this control as the intestine. This will be further discussed in section 1.3.3.

1.3.2.3 The effects of 1,25-dihydroxyvitamin D_3 on calcium transport in the kidney. The effects of $1,25\text{-}(OH)_2D_3$ on renal calcium transport are not clearly established. Some found a decrease in the amount of urinary calcium following the administration of $1,25\text{-}(OH)_2D_3$, while others have found either no effect or even an increase in urinary calcium excretion (Puschett et al, 1972; Burnatowska et al, 1985). Stimulatory effects of $1,25\text{-}(OH)_2D_3$ on phosphate reabsorption are only noted in the presence of PTH (Nseir et al, 1978). Kurnik and Hruska (1985) have suggested that the effect of $1,25\text{-}(OH)_2D_3$ is mediated by changes in the lipid composition of the luminal membrane of the tubular cell.

1.3.2.4 The effect of 1,25-dihydroxyvitamin D_3 on the parathyroid gland.
Calcium is the classical effector of PTH secretion such that plasma calcium and PTH levels are inversely correlated (Sherwood et al, 1966). More recent studies demonstrated the presence of $1,25\text{-}(OH)_2D_3$ receptors in the parathyroids, suggesting that $1,25\text{-}(OH)_2D_3$, the production of which is stimulated by PTH, may have a direct negative feedback inhibition on PTH secretion.

Using molecular probes, several investigators have confirmed this suggestion (Cantley et al, 1985; Silver et al, 1985; Russell et al, 1986). It was shown that $1,25\text{-}(OH)_2D_3$ suppresses the levels of mRNA for the synthesis of preproPTH, the biosynthetic precursor of PTH. This effect is specific, dose-dependent and fully reversible. Even at $1,25\text{-}(OH)_2D_3$ concentrations of 10^{-11} M a suppressive effect on PTH synthesis was noted. Slatopolsky et al (1984) have been able to demonstrate that intravenous $1,25\text{-}(OH)_2D_3$ can also reduce the release of PTH in humans significantly without apparently affecting the level of serum calcium.

1.3.3 Bone formation, mineralization and remodelling
A deficiency of vitamin D impairs the mineralization of bone which produces in children rickets and in adults osteomalacia. In both instances the bone matrix collagen fails to mineralize and is, therefore, unable to carry out the mechanical function of the skeleton. In young individuals this results, due to weight bearing stress and muscle function, in bending of the bones and the so-called 'green-stick' fractures. In adults failure of mineralization only occurs at sites where remodelling takes place. At these sites large seams of unmineralized matrix (osteoid) are found. In longstanding disease in adults some bending of long bones may be seen also.

In vitamin D-deficient animals bone matrix synthesis has been reported to be reduced (Baylink et al, 1970; Barnes et al, 1973). Although treatment with vitamin D restores matrix synthesis and mineralization, it is not clear whether the latter is a

direct effect or an indirect effect due to stimulation of intestinal calcium and phosphorus absorption (Howard and Baylink, 1980).

In vitamin D-deficient rats it was shown that vitamin D, and more specifically 1,25-$(OH)_2D_3$, is not necessary for a normal skeletal development and mineralization (Underwood and DeLuca, 1980; Brommage et al, 1984; Weinstein et al, 1984). Studies in humans are scanty, however. Popovtzer et al (1973) previously showed that calcium and phosphorus, intravenously (iv) administered on alternate days, resulted in the cure of osteomalacia in two adult patients. Vitamin D metabolites were not determined in this study, making it impossible to exclude a permissive effect of low 1,25-$(OH)_2D_3$ levels. Balsan et al (1986) recently reported that treatment of a child with heriditary resistance to 1,25-$(OH)_2D_3$ with calcium iv led to correction of biochemical abnormalities and healing of rickets. At present this is the only study available, showing evidence that in humans normal mineralization can be achieved in the absence of a normal 1,25-$(OH)_2D_3$ receptor-effector system, provided that normal serum calcium and phosphate levels are maintained. However, possible non-receptor-mediated effects of 1,25-$(OH)_2D_3$ (Rasmussen et al, 1982; Nemere et al, 1984; Lieberherr et al, 1987) can not be excluded, at the high circulating levels of the active vitamin D metabolite, in children with heriditary resistance to 1,25-$(OH)_2D_3$.

Matsumoto et al (1985) observed an 1,25-$(OH)_2D_3$-induced increase in phosphatidylserine (PS) synthesis in cloned osteogenic sarcoma cells. Because PS is thought to be important for apatite formation and bone mineralization by binding of calcium and phosphate to form calcium-PS-phosphate complexes (Cotmore et al, 1971; Majeska et al, 1979), it was suggested that 1,25-$(OH)_2D_3$ might also stimulate bone mineralization by a direct effect on osteoblasts.

Osteoblasts are the only bone cells possessing 1,25-$(OH)_2D_3$ receptors (for review Haussler, 1986; Nijweide et al, 1986). Direct effects of 1,25-$(OH)_2D_3$ on osteoblast-like sarcoma cells include enhanced osteocalcin biosynthesis (Price and Baukol, 1980) and alkaline phosphatase activity (Manolagas et al, 1981; Majeska and Rodan, 1982). On the other hand, in primary cultures of mouse osteoblast-like (OB) cells, alkaline phosphatase activity was found to be inhibited (Wong et al, 1977). Similar conflicting results were obtained with regard to effects of 1,25-$(OH)_2D_3$ on cell proliferation (Chen et al, 1983; Herrmann-Erlee et al, 1985). Although in most studies, using rat calvaria, a 1,25-$(OH)_2D_3$-induced inhibition of collagen synthesis by osteoblasts has been found (Rowe and Kream, 1982), recently a stimulatory effect of 1,25-$(OH)_2D_3$ has been reported in osteoblast-like cells of other species, including man (Kurihara et al, 1984; Beresford et al, 1986). Some investigators suggested that all these conflicting results could be explained by differences in the state of phenotypic differentiation of the OB cells in culture (Spiess et al, 1986). In this respect it is important to note that it is not known whether new bone formation is the result of activation of resting osteoblasts or of the proliferation and differentia-

tion of preosteoblasts. Also the exact function of proteins like osteocalcin and alkaline phosphatase in bone formation and mineralization has not been characterized.

1,25-$(OH)_2D_3$ also seems to induce changes in the cytoskeleton of rat OB cells and rat osteosarcoma cells (Gronowicz et al, 1986). Such morphological changes in osteoblasts may play a role in bone resorption (Rodan and Martin, 1981). For instance retraction and rounding of the cells may expose bone or osteoid surface and initiate osteoclastic bone resorption (Rodan and Rodan, 1984; Chambers, 1985). Whether there is also a relationship with the production of collagenase by osteoblasts remains to be established (Sakamoto and Sakamoto, 1986).

Of the several factors known to affect bone resorption, one of the most potent is 1,25-$(OH)_2D_3$. In vitro, 1,25-$(OH)_2D_3$ alone can stimulate bone resorption (Raisz et al, 1972), whereas in vivo PTH and 1,25-$(OH)_2D_3$ work in concert to mobilize bone mineral (Garabedian et al, 1974). 1,25-$(OH)_2D_3$ is proposed to stimulate bone resorption, at least in part, by promoting the differentiation of osteoclast progenitors and, thereby, raising the number of mature osteoclasts (Bar-Shavit et al, 1983; Merke et al, 1986; Provvedini et al, 1986). Others have suggested that 1,25-$(OH)_2D_3$ could also indirectly enhance osteoclastic bone resorption by its effect on lymphokine(s) production by T lymphocytes (Abe et al, 1983; Amento et al, 1984; Haussler, 1986). For instance, a promoting effect of 1,25-$(OH)_2D_3$ on the action of interleukin-I, a strong bone resorbing agent (Gowen et al, 1983), has been proposed (Haussler, 1986). Finally, 1,25-$(OH)_2D_3$ could modulate bone resorption through interaction with other systemically (e.g. PTH) and locally produced bone resorbing agents (see Chapter 3).

In conclusion, 1,25-$(OH)_2D_3$ seems to play a role in bone remodelling by a complex action on both osteoblasts and osteoclasts. In bone formation its effects seem paradoxical. In vitro the hormone inhibits collagen synthesis in most model systems, but it is necessary for adequate bone matrix formation and mineralization in vivo. A teleological explanation, proposed by Raisz and Kream (1983), is that the catabolic effects of 1,25-$(OH)_2D_3$ are useful when exogenous supply of calcium and phosphorus is insufficient. When intake is adequate, the major function of 1,25-$(OH)_2D_3$ is to stimulate intestinal absorption of calcium and phosphate. That this may be a too simple explanation of the action of 1,25-$(OH)_2D_3$ is illustrated by the stimulatory action of 1,25-$(OH)_2D_3$ on the collagen synthesis (at mRNA level) by human bone-derived osteoblasts (Beresford et al, 1986).

1.3.4 Cell proliferation and differentiation

1,25-$(OH)_2D_3$, acting via its receptor protein, has been shown to be a potent regulator of the differentiation and proliferation of a variety of cell types (for reviews Eisman, 1984; Braidman and Anderson, 1985; Suda et al, 1984, 1986).

In some cases inhibition of cell growth is coupled to morphological changes

(Frampton et al, 1983; Dokoh et al, 1984; Gronowicz et al, 1986) and differentiation on the cells (Abe et al, 1981; Bar Shavit et al, 1983; Spiess et al, 1986). Especially during the last five years the effect of 1,25-$(OH)_2D_3$ on the cells of the immune system has been extensively studied. The overall effect of 1,25-$(OH)_2D_3$ on monocytes and related cell types appears to be to inhibit the proliferation of immature precursors and to enhance differentiation to mature cells (Abe et al, 1981; Tanaka et al, 1982, 1984; Bar Shavit et al, 1983; Suda et al, 1984; Manolagas et al, 1986). Several investigators found that the activation of both T and B lymphocytes with mitogenic lectins, antigens and viruses leads to the expression of the receptor protein for 1,25-$(OH)_2D_3$ (Provvendi et al, 1983; Manolagas et al, 1985). Manolagas et al (1986) also found, that the 1,25-$(OH)_2D_3$-induced inhibition of proliferation of human peripheral blood mononuclear cells is mediated through a selective inhibition of interleukin-2 production. Furthermore, 1,25-$(OH)_2D_3$ also markedly inhibits gamma-interferon production by activated human T lymphocytes (Rigby et al, 1987). The gamma-interferon stimulated synthesis of 1,25-$(OH)_2D_3$ in macrophages (Koeffler et al, 1985) may, therefore, represent a negative feed-back loop on T cell proliferation and gamma-interferon production.

Treatment of haemodialyzed patients with 1α-$(OH)D_3$ enhanced the in vitro lymphoproliferative response to mitogens, without influencing lymphocyte counts or the ratio of lymphocyte subpopulations (Tabata et al, 1986). This observation suggests once more a role for 1,25-$(OH)_2D_3$ in cellular immunity, and adds a new link between the endocrine and the immune system as already exemplified by the immunosuppressive actions of glucocorticoids.

One of the ways by which 1,25-$(OH)_2D_3$ can reduce cell proliferation and enhance differentiation could be through inducing changes in the intracellular calcium concentration and/or decreased transcription of oncogenes. The finding that changes in extracellular calcium considerably influenced the action of 1,25-$(OH)_2D_3$ on murine leukemia cells, supports the former possibility. Whether induction of CaBP or changes in cellular calcium transport are involved is presently unknown.

The linkage between changes in oncogene expression and the ultimate effect of 1,25-$(OH)_2D_3$ on cell growth and maturation was for the first time suggested by Reitsma et al (1983). These investigators showed that 1,25-$(OH)_2D_3$ reduces C-myc mRNA levels in promyelocytic leukemia cells (HL-60) within 4 h of exposure to the hormone and that these changes precede the onset of other phenotypic changes by a minimum of 8 additional hours. Also inhibitors of lymphocyte proliferation, like cyclosporin A, seem to exert their antiproliferative effect by decreasing the levels of C-myc oncogene mRNA (Reed et al, 1985). Manolagas et al (1987) recently presented data, showing a close association between the expression of C-myc oncogene mRNA and the expression of the receptor protein for 1,25-$(OH)_2D_3$ in 3T3 fibroblasts. Taken together the above cited studies suggest that 1,25-$(OH)_2D_3$, acting via its recep-

tor protein, might be a natural counter-regulatory signal in oncogene-modulated cell proliferation and (de)differentiation. Furthermore, the expression of 1,25-$(OH)_2D_3$ receptors in a wide variety of cells (Table II) could be the result of oncogene activation in these cells. Whether the effects of 1,25-$(OH)_2D_3$ can be exploited in the treatment of certain malignancies (Suda et al, 1984) is open to question.

1.3.5 Endocrine tissues

Investigations into the effects of 1,25-$(OH)_2D_3$ on endocrine tissues have concentrated upon the relationship between 1,25-$(OH)_2D_3$ receptors, the presence of CaBP and changes in hormone secretion (for review Henry and Norman, 1984; Braidman and Anderson, 1985). Several studies, mainly using rat and chicken tissue, have demonstrated the presence of both 1,25-$(OH)_2D_3$ receptors and CaBP in the β-cell of the pancreas, specific cells of the pituitary and cells of the parathyroid glands (Christakos et al, 1979; Jande et al, 1982; Strumpf et al, 1979; Lawson et al, 1984).

Norman et al (1980) reported that the stimulation of perfused pancreatic tissues from vitamin D-deficient rats with glucose or arginine hydrochloride led to little insuline secretion. Repletion of these animals with vitamin D enhanced insuline secretion from the isolated pancreas, while glucagon secretion remained unaffected. In clonal rat pituitary cells of the GH_4C_1 strain it was shown that 1,25-$(OH)_2D_3$ raises the relative amount of prolactin mRNA and that the expression of this effect is dependent on extracellular calcium (Wark and Tashjian, 1983). Also changes in mRNA levels for calcitonin and preproPTH in response to 1,25-$(OH)_2D_3$ have been reported (Segond et al, 1985; Silver et al, 1985),

In combination with the simultaneous presence in these tissues of 1,25-$(OH)_2D_3$ receptors and CaBP, the results suggest that 1,25-$(OH)_2D_3$, by analogy with its effects in the intestinal mucosa, induces the synthesis of CaBP. This may facilitate the delivery of calcium-mediated signals to sites regulating hormone production and release. However, in children with hereditary resistance to 1,25-$(OH)_2D_3$ (defective receptor-effector system), Hochberg et al (1985) found no significant abnormalities in hormone secretion from the pituitary, pancreas and testis, apart from those presumably due to hypocalcemia. Although these findings challenged the above-mentioned hypothesis, there is still the possibility that 1,25-$(OH)_2D_3$ has two different modes of action in endocrine glands. One, through a putative direct effect on calcium transport (Rasmussen et al, 1981; Lieberherr et al, 1986), and the other, through the interaction of 1,25-$(OH)_2D_3$ with its receptor and consequently with the biosynthesis of new mRNA for the different hormones. Especially the effect of 1,25-$(OH)_2D_3$ on calcium transport, which is claimed to be a non-receptor mediated event, may be involved in the regulation of hormone release.

In conclusion, the effects of 1,25-$(OH)_2D_3$ on endocrine tissues is a new exciting field of research. However, it would be premature to relate the present findings to

a general physiological role of 1,25-$(OH)_2D_3$ in hormone secretion by endocrine glands.

CHAPTER 2

Scope of this thesis

The data published on the action of 1,25-$(OH)_2D_3$ on growth and phenotypic expressions of osteoblast-like cells (OB-cells) are conflicting (Chapter 1, section 1.3.3). Especially, contradictory results have been obtained with regard to the effect of 1,25-$(OH)_2D_3$ on alkaline phosphatase (AP) activity. Majeska and Rodan (1982) have proposed a unifying explanation for these apparently conflicting experimental findings. They suggest that 1,25-$(OH)_2D_3$ may either enhance or inhibit AP, depending on the state of growth and maturation of the cells. *One of the aims* of the present thesis was to investigate the effect of 1,25-$(OH)_2D_3$ on growth and maturation (differentiation) in low and high density cultures of rat OB cells. As a parameter of maturation we used AP activity. Although the precise function of this enzyme is still not understood, it has been clearly established that AP is mainly found in OB cells and that mature osteoblasts have the highest activity. Under the same experimental conditions we also studied the effect of 1,25-$(OH)_2D_3$ on the adenylate cyclase system in an attempt to further characterize the previously reported attenuation of the cyclic AMP response to PTH by the active hormonal form of vitamin D (Wong et al, 1977).

It is well established that many cell types possess receptors for 1,25-$(OH)_2D_3$ and respond to the sterol by changes in their biological activity. It seems improbable that the ability of such a wide variety of tissues to respond to 1,25-$(OH)_2D_3$ relies solely on the synthesis of 1,25-$(OH)_2D_3$ in the kidneys. In this regard several mechanisms to regulate the 1,25-$(OH)_2D_3$ concentration and activity at the level of the target tissue could be postulated. One possibility, as reviewed in Chapter 1 (section 1.1.3), is that the bioactive sterol is formed in extrarenal sites. The observation, that in anephric patients some investigators observed low but still detectable levels of 1,25-$(OH)_2D_3$, also supports this hypothesis (Lambert et al, 1981; Jongen et al, 1981). Other possible mechanisms at the cellular level which could provide a means for regulation of the response to the sterol are: ligand dependent regulation of the number of receptors or regulation of the cellular 1,25-$(OH)_2D_3$ level through the ability of the hormone to induce its own metabolism (Chapter 1, section 1.1.5.2). Based on these three hypotheses *the second aim* of our studies was to investigate the significance of vitamin D metabolism and 1,25-$(OH)_2D_3$ receptor regulation in osteoblast-like cells as possible mechanisms for the control of vitamin D action at the cellular level (Chapter 3, section 3.3.3 and Chapter 4). Special attention was given to the relation between the self-induced metabolism of 1,25-$(OH)_2D_3$ and receptor regulation by the active metabolite of vitamin D_3.

Since the discovery that vitamin D enhances calcification of undermineralized

bone the mechanism of its skeletal action has been extensively studied. Recently considerable evidence has accumulated to suggest that 1,25-$(OH)_2D_3$ can influence bone cell function (Chapter 1, section 1.3.3; Chapter 3), supporting a direct influence of the hormone on bone formation and/or mineralization. On the other hand it has been shown that intravenous administration of calcium (and phosphate) can be successfully used to treat the osteomalacia due to vitamin D deficiency and vitamin D-dependent rickets type II (Chapter 1, section 1.3.3). Therefore, it remains to be elucidated whether 1,25-$(OH)_2D_3$ promotes bone mineralization directly or indirectly, i.e. through enhancing the circulating levels of calcium and phosphate. *The third aim* of the present thesis was to determine the influence of the subnormal availability of 1,25-$(OH)_2D_3$ in patients with renal failure on bone turnover and mineralization and to study the histomorphometrical changes after treatment with 1α-$(OH)D_3$ for three months. Special emphasis was given to the interrelationships between the results of various histomorphometric measurements. Also, these results were correlated with biochemical parameters in an attempt to differentiate between possible direct and indirect effects of the treatment-induced rise of 1,25-$(OH)_2D_3$ levels on the skeletal manifestations of renal osteodystrophy.

CHAPTER 3

Properties of cultured osteoblast-like cells in relation to vitamin D

3.1 Introduction

In this chapter both the response of osteoblast-like cells (OB cells) to 1,25-$(OH)_2D_3$ and the ability of these cells to metabolize 25-$(OH)D_3$ to 1,25-$(OH)_2D_3$ will be discussed.

With regard to the effects of 1,25-$(OH)_2D_3$ on cell proliferation and differentiation we have examined the growth response and changes in alkaline phosphatase (AP) activity in low and high density cultures. This approach was chosen in order to ascertain whether responses differ due to cell density and/or proliferation rate (section 3.3.1).

Although other investigators have already shown that treatment with 1,25-$(OH)_2D_3$ resulted in an attenuation of the cyclic AMP (cAMP) response to parathyroid hormone (PTH) in rat OB cells (Chen et al, 1984), it is not known whether this is specific for the PTH-stimulated cAMP response or represents a more general effect on the adenylate cyclase system. Therefore, we have used different agents to stimulate the adenylate cyclase system in an attempt to clarify the role of 1,25-$(OH)_2D_3$ in the modulation of this system and to obtain information about its mode of action (section 3.3.2).

Extrarenal production of 1,25-$(OH)_2D_3$ has been shown to occur in a number of tissues (section 1.1.3). Because of the intimate interaction between bone metabolism and 1,25-$(OH)_2D_3$, the skeleton seems a possible site for extrarenal production of the active hormone of vitamin D. In the last section (3.3.3) of this chapter we report the expression of 25-$(OH)D_3$ metabolizing enzymes, including 1α-hydroxylase, in OB cells derived from fetal chicken calvaria.

3.2 Materials and methods

Cell culture

Rat OB (ROB) cells: see appendix paper 1.

Chicken OB (COB) cells: see appendix paper 2.

UMR-106 cells: Rat osteogenic sarcoma cells (UMR-106), provided by Dr. MPM Herrmann-Erlee (Dept. of Cell Biology and Histology, University of Leiden, Leiden, The Netherlands) were cultured in αMEM medium with 10% fetal calf serum (FCS), penicilline (100 U/ml), streptomycine (100 ug/ml) in a humified 95% air – 5% CO_2

atmosphere and at the cells densities indicated. After 24 h, the medium was replaced by αMEM with 2% charcoal-treated serum. The further procedure was identical to that described for primary cultures of ROB cells.

Human bone cells: The method used was adapted from Beresford et al (1984). Specimens of human trabecular bone, obtained at orthopedic surgery, were extensively washed in Hanks buffered salt solution (BSS), pH 7.4, and then dissected into small fragments (0.1-0.5 cm). After an additional wash in Hanks BBS, trabecular fragments were seeded as explants into culture flasks (75 cm) and cultured in αMEM with 10% FCS and the usual additives in a humified atmosphere of 95% air and 5% CO_2. The medium was replaced at 5-7 days intervals. Outgrowth of cells occurred after approximately 7-14 days and the cultures were confluent after approximately 4-5 weeks. At confluence the cells were harvested after trypsinization (trypsine 0.05%, EDTA 0.02%), and suspended at 10.10^3 cells/cm^2 in petri dishes (35 mm). After 24 h in αMEM, 10% FCS, the medium was replaced by αMEM with 2% charcoal-treated serum. The further procedure is identical to that described for ROB cells (appendix paper 1).

Assays

DNA content, AP activity and cAMP: see appendix paper 1.

[^{3}H]thymidine incorporation: According to the method of Van der Plas et al (1985), the cells were pulsed prior to harvest with 37 KBq/ml [^{3}H]thymidine for 2 h. The pulse incubation was stopped by placing the cells on ice and washing three times with ice-cold Hanks BSS. To remove free intracellular radioactive label, cells were lysed in distilled water (15 min, 0°C). In order to determine the [^{3}H]thymidine incorporation into DNA, the cell residues were trypsinized (trypsine 0.05%, EDTA 0.02%) and sonicated. Aliquots were taken for direct scintillation counting.

25-(OH)D_3 and 1,25-$(OH)_2D_3$ metabolism: see appendix paper 2 and 3.

3.3 Results and discussion

3.3.1 The effects of 1,25-$(OH)_2D_3$ on alkaline phosphatase activity and growth of osteoblast-like cells

Alkaline phosphatase (AP) is a phenotype-specific product of osteoblast-like cells (OB cells) with the highest activity of this enzyme being found in mature osteoblasts (Robinson et al, 1973). The precise role of AP in the function of the osteoblast is not known. Most theories suggest that the enzyme might be involved in mineralization by increasing local phosphate ion concentration and/or by hydrolyzing inhibitors of crystallization such as pyrophosphate (Boskey, 1981; Wuthier, 1982).

The reports concerning the effects of 1,25-$(OH)_2D_3$ on the AP activity of OB cells have been conflicting. In primary cultures of neonatal mouse OB cells, an inhibitory

effect of 1,25-$(OH)_2D_3$ has been found (Wong et al, 1977), while a stimulation of AP activity was shown in the cloned mouse OB cell line MC3T3-E1 (Kurihara et al, 1984). Stimulatory effects of the sterol on AP have also been observed in rat and human osteosarcoma cell lines (Manolagas et al, 1981; Mulkins et al, 1983) and proliferating, but not confluent cultures of neonatal ROB cells (Brandt et al, 1982).

In primary cultures of fetal ROB cells, we found after three days of culture in the presence of 1,25-$(OH)_2D_3$ a consistent stimulatory effect of the sterol on AP activity (appendix paper 1). The minimum effective dose was 10^{-10} M, consistent with physiological concentrations of the hormone. In human-derived cells similar results were obtained (Fig. 1) suggesting that ROB cells, at least with regard to AP activity, behave in the same way as human OB cells in response to 1,25-$(OH)_2D_3$.

To investigate whether the reported discrepancies in the effect of 1,25-$(OH)_2D_3$ on

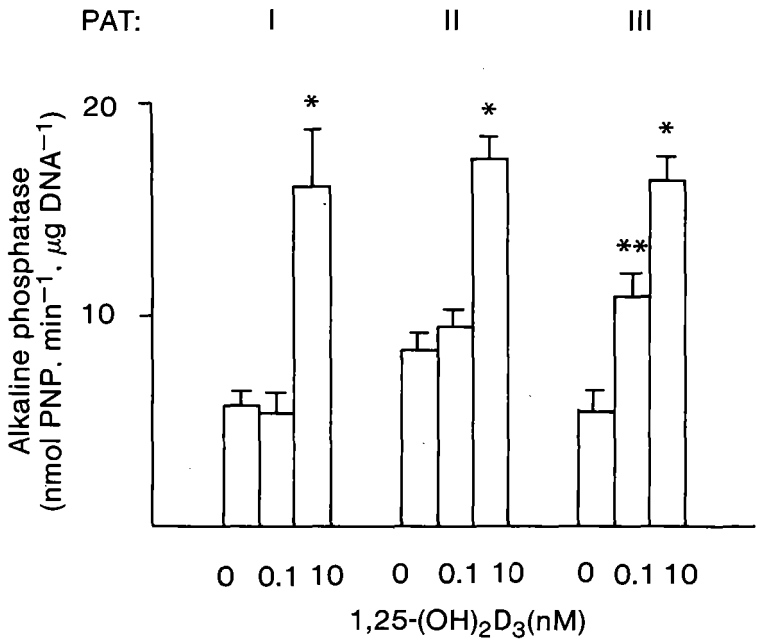

*Fig. 1: Effect of 1,25-$(OH)_2D_3$ on AP activity of human bone cell cultures. Cells were incubated for 3 days in the absence or presence of 1,25-$(OH)_2D_3$ at the indicated concentrations. Medium (αMEM with 2% charcoal-treated FCS) was changed at 48 h. Experiments were performed at first passages (for details see Methods). The donors were a 67 yr old female with cox arthrosis (Pat I), a 59 yr old female with cox arthoris (Pat II), and a 46 yr old male with M. Perthes of the right femur (Pat III). Specimens of trabecular bone were obtained during orthopedic surgery. The data are expressed as means ± SD of 4-5 cultures. *$p<0.01$, **$p<0.05$ versus control.*

AP activity could be explained by methodological differences, we studied the effect of 1,25-$(OH)_2D_3$ on the enzyme at different ROB cell densities and culture conditions. During the first days of culture a positive correlation between initial cell density and AP activity was found, and irrespective of cell density a higher AP level in the cultures subsequently treated with 1,25-$(OH)_2D_3$ (Fig. 2). However, dependent upon

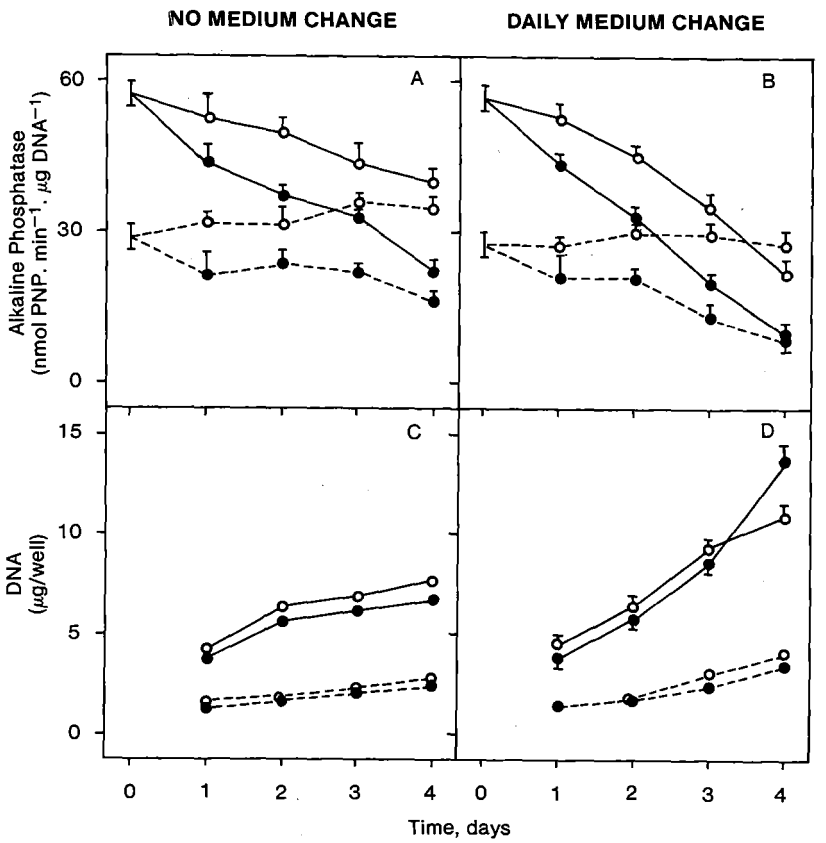

Fig. 2: A typical time-course of the effect of 1,25-$(OH)_2D_3$ (10^{-8} M) on AP activity and DNA content in sparsely and densely seeded ROB cell cultures at different culture conditions. ***Left panels*** *(A,C): no change of the medium (αMEM with 2% charcoal-treated FCS); addition of vehicle (●) and 1,25-$(OH)_2D_3$ (○) only at the start of the experiment.* ***Right panels*** *(B,D): daily change of medium and daily addition of vehicle (●) or 1,25-$(OH)_2D_3$ (○).* ***Upper panels*** *(A,B): AP activity in nmol PNP.min^{-1}.ug DNA^{-1}.* ***Lower panels*** *(C,D): DNA content. Sparsely (5.10^3 cells/cm^2) seeded cultures (--); Densely (20.10^3 cells/cm^2) seeded cultures (—). The data are expressed as means ± SD of 4 cultures.*

both cell density and culture conditions (daily versus no change of medium) a rapid loss of AP activity (expressed per ug DNA) during culture was found. Due to this phenomenon also the correlation between AP and cell density was ultimately lost.

The loss of the AP phenotype in primary culture systems could be related to several factors such as cell membrane alterations induced by collagenase digestion, the artificial environment during culture and selective proliferation of cells that do not possess the AP phenotype (Wolffe and Tata, 1984). The last mentioned possibility could be responsible for the loss of AP activity as observed in the densely seeded cultures. Both exogenous factors like the daily change of medium and the secretion in the medium of endogenously produced growth factor(s) (Wong et al, 1986, 1987b) could have stimulated the growth of undifferentiated mesenchymal cells in these densely seeded cultures. On the other hand, Fritsch et al (1985) showed that in low density cultures treatment with 1,25-$(OH)_2D_3$ resulted in a maintenance of AP activity and suggested that the loss of enzyme activity in the control cultures was not due to selective proliferation. Our results in sparsely seeded cultures are in accordance with this observation. Therefore, we suggest that 1,25-$(OH)_2D_3$ acts as an agent that prevents the dedifferentiation of osteoblasts in primary culture and that in rapidly proliferating cultures this effect is less pronounced, probably due to an increased selective proliferation of less differentiated OB cells.

In rat osteogenic sarcoma cells (ROS 17/2.8) Majeska and Rodan (1982) observed pleiotrophic effects of 1,25-$(OH)_2D_3$ on AP activity dependent upon growth and cellular maturation. Thus, in low density cultures the effect of 1,25-$(OH)_2D_3$ was stimulatory, whereas in high density cultures the hormone inhibited enzyme activity. In agreement with the findings in ROS 17/2.8 cells, we found that basal AP activity in both normal rat OB cells (Fig. 2), but also in osteogenic sarcoma cells (UMR-106, Table I) was positively correlated with cell density. At all densities examined, however, the effect of 1,25-$(OH)_2D_3$ on AP activity of normal rat OB cells (Fig. 2) remained stimulatory compared to control cultures. In UMR-106 cells, on the other hand, we found neither a stimulatory nor an inhibitory effect of 1,25-$(OH)_2D_3$ on the enzyme (Table II). Especially this last finding raises the question whether osteosarcoma cells are a suitable model system to study the effects of 1,25-$(OH)_2D_3$ on cellular maturation of osteoblasts. A recent study in subclones of the ROS 17/2.8 cell line showed lack of osteocalcin production in phenotypes with high AP levels (Spiess et al, 1986), which suggests that such cells may be in a dedifferentiated state since they have lost the ability to express one of the osteoblast phenotype-specific products. Furthermore, it was shown that in these dedifferentiated cells 1,25-$(OH)_2D_3$ inhibited AP activity by a 1,25-$(OH)_2D_3$ receptor independent mechanism. Therefore, the suggestion that the inhibitory effect of 1,25-$(OH)_2D_3$ on the AP activity of the ROS 17/2.8 line is exerted in phenotypically mature osteoblasts (Majeska and Rodan, 1982), as defined by their high AP activity, seems ques-

Table I: Effect of 1,25-$(OH)_2D_3$ on DNA content and AP activity in UMR-106 cells, seeded at different densities.

	1,25-$(OH)_2D_3$ concentration					
	Control		10^{-10} M		10^{-8} M	
Plating density (cells/cm²)	DNA	AP	DNA	AP	DNA	AP
2.5 x 10^3	9.6±0.6	121±6	8.9±0.4	133± 7	8.7±0.1	128±14
5 x 10^3	12.5±0.4	169±4	12.0±0.2	186±12	11.4±0.4	184±15
10 x 10^3	17.3±1.1	225±25	15.4±0.8	223±27	14.5±1.0*	242±18

Cells were incubated for 3 days in the absence or presence of 1,25-$(OH)_2D_3$ at the indicated concentrations. Medium (αMEM with 2% charcoal-treated FCS) was changed at 48 h. AP is expressed in nmol PNP.min^{-1}.ug DNA^{-1} and DNA in ug/well. Values are means ±SD of 5 cultures. *$p < 0.05$ versus control.

tionable. It is more likely that dedifferentiation of cells explains the contradictory results obtained in osteosarcoma cell lines.

The results of experiments in which radiolabelled thymidine incorporation was used as an index of DNA synthesis showed that the proliferative response of ROB cells to 1,25-$(OH)_2D_3$ was dependent upon cell density (Fig. 3). In sparsely seeded cultures we found a significant increase in [^{3}H]thymidine incorporation, while this was decreased in densely seeded cultures. However, using DNA content as a measure of cell growth an antiproliferative effect of the hormone was only observed in high-density cultures (appendix paper 1). Similar experiments by Chen et al (1983) showed that 1,25-$(OH)_2D_3$ inhibited cell proliferation, irrespective of cell density. A methodological difference that might be important concerns the daily change of medium. Recently, it was shown that serum factors, but also 1,25-$(OH)_2D_3$ contribute to ROB cell adhesion to culture wells (Fritsch et al, 1985). The mechanical stress through the daily change of medium could have resulted in a loss of attached cells, especially in the 1,25-$(OH)_2D_3$ depleted sparsely seeded cultures. A lower [^{3}H]thymidine incorporation in the control cultures could have been the consequence. Anyway, the fact that in low density cultures 1,25-$(OH)_2D_3$ treatment results in a maintenance of AP activity, while proliferation is not clearly inhibited, suggests that the effect of 1,25-$(OH)_2D_3$ on AP is not linked to an antiproliferative effect of the hormone. Furthermore, it appears that for the growth inhibition by 1,25-$(OH)_2D_3$ cell-cell interactions are essential, because this phenomenon was only observed in densely seeded cultures of both normal ROB cells and osteosarcoma cells (Table I).

For the in vivo situation the relevance of the effects of 1,25-$(OH)_2D_3$ on growth

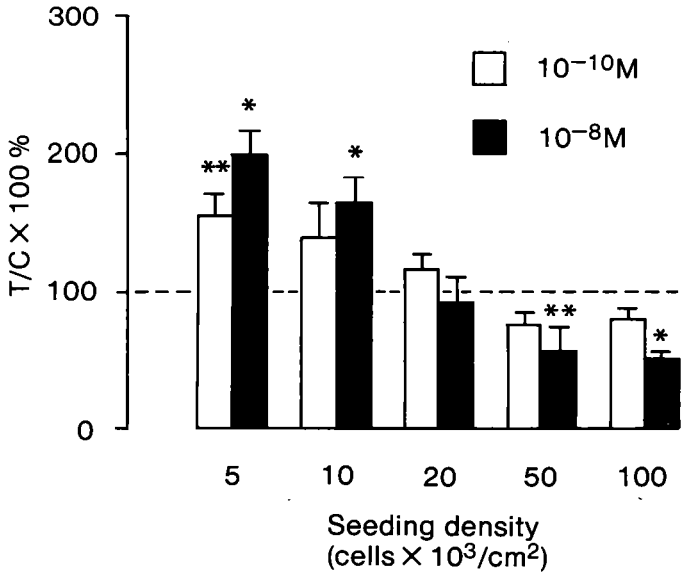

*Fig. 3: The effect of 1,25-$(OH)_2D_3$ on the proliferation of rat OB cells seeded at different cell densities. Cells were seeded in αMEM with 10% FCS at the indicated densities. After 24 h the medium was changed to αMEM with 2% charcoal-treated FCS with or without 1,25-$(OH)_2D_3$ (10^{-10}, 10^{-8}M). The medium was changed daily. After 3 days [^{3}H]thymidine incorporation was measured during the last 2 h of culture as described in Methods. The results are expressed as the ratio of treated over control (T/C) × 100%. Data are means ± SD of 4-6 cultures. *$p < 0.01$, **$p < 0.05$ versus control.*

of OB cells remains speculative. However, the fact that 1,25-$(OH)_2D_3$ inhibits the growth of densely seeded cultures might bear relevance to the effects of the hormone in renal osteodystrophy (Chapter 5). In this metabolic bone disease, with high bone turnover and, consequently, an increased number of osteoblasts, we found a significant decline in osteoblast seams percentage after short-term treatment with 1α-$(OH)D_3$ (Chapter 5).

From the results presented and the data from the literature it seems apparent that 1,25-$(OH)_2D_3$ may be an important regulator of growth and differentiation of osteoblasts. The data also support the concept that 1,25-$(OH)_2D_3$ through an effect on the maturation of osteoblast precursors might affect bone formation in vivo. The failure to demonstrate a significant effect of 24,25-$(OH)_2D_3$ (appendix paper 1) does not preclude a role for this metabolite in skeletal homeostasis, but suggests that its action, if any, is distinct from that of 1,25-$(OH)_2D_3$.

3.3.2 The effect of 1,25-dihydroxyvitamin D_3 on the adenylate cyclase in rat osteoblast-like cells

Although in many respects 1,25-$(OH)_2D_3$ behaves as a steroid hormone, in some targets its physiological actions are similar to those of hormones which act through cAMP. This is the case in bone, in which both 1,25-$(OH)_2D_3$ and PTH promote bone resorption (Raisz et al, 1972). However, in primary cultures of mouse OB cells Wong et al (1977) showed that the activity of 1,25-$(OH)_2D_3$ clearly differs from that of PTH; the steroid did not induce an increased cAMP generation, whereas PTH did. The same investigators reported that pretreatment of the cultures with 1,25-$(OH)_2D_3$ made the OB cells refractory to the effect of PTH on cAMP. This last observation was confirmed in later studies in primary cultures of ROB cells (Chen and Feldman, 1984) and clonal osteogenic sarcoma cells (Catherwood, 1985). Our data obtained with ROB cells also support the concept that 1,25-$(OH)_2D_3$ treatment results in an attenuation of the cAMP response to PTH (appendix paper 1).

The adenylate cyclase system is believed to encompass a complex cascade mechanism with stimulatory and inhibitory limbs depending on the cell type (Gilman, 1984). To more directly observe 1,25-$(OH)_2D_3$ regulation of this system at sites beyond the PTH receptor, we used forskolin. As outlined in detail in appendix paper 1, the results obtained with this agent suggest that 1,25-$(OH)_2D_3$ affects the adenylate cyclase at the level of the nucleotide regulatory protein (N) or through an effect on the interaction of N with the catalytic unit. Studies in osteosarcoma cells also indicate that the principal action of 1,25-$(OH)_2D_3$ is at or near the N protein (Catherwood, 1985; Rizzoli and Fleisch, 1986). Studies carried out to determine whether enhanced activity of the inhibitory N protein (N_i) could be responsible for the effect of 1,25-$(OH)_2D_3$ did not provide evidence in favour of an alteration in N_i activity (Kubota et al, 1985; Pines et al, 1986). Conflicting data exist regarding the interaction of 1,25-$(OH)_2D_3$ with the stimulatory N protein (N_S). Kubota et al (1985) reported that 1,25-$(OH)_2D_3$ treatment of osteogenic sarcoma cells did not result in a loss of total N_S activity, as measured by reconstitution of stimulated adenylate cyclase activity of UMR-106 cells in N_S deficient membrane preparations of cyc-S49 mouse lymphoma cells. On the other hand the lower response to specific N_S agonists (NaF and guanosine triphoshate) in 1,25-$(OH)_2D_3$ treated ROS 17/2.8 sarcoma cells suggests a reduced activity of the N_S (Rizzoli and Fleisch, 1986).

A logical consequence of a mechanism of action of 1,25-$(OH)_2D_3$ beyond the PTH receptor is that also the cAMP response to other hormonal agonists of the adenylate cyclase is attenuated. Indeed an attenuation of isoproterenol stimulated cAMP production has been demonstrated in 1,25-$(OH)_2D_3$ treated osteosarcoma cells (Kubota et al, 1985; Catherwood, 1985), while also heterologous desensitization to calcitonin and prostaglandin E_2 (PGE_2) has been reported (Wong et al, 1977; Kent et al, 1980; Kubota et al, 1985). In UMR-106 cells we also found a 1,25-$(OH)_2D_3$-induced at-

Table II: The effect of 1,25-$(OH)_2D_3$ on cAMP generation by different agonists.

	cAMP (pmol/10 ug DNA)	
AGONIST	Control	1,25-$(OH)_2D_3$
PTH	290 ±9	205 ±14*
Isoproterenol	19.2±2.2	43.6±3.6*
PGE_2	4.1±0.8	5.2±0.4

ROB cells were treated with 1,25-$(OH)_2D_3$ (10^{-8}M) for 2 days. Cells were seeded at an initial density of 20.10^3 cells/cm^2. Medium (αMEM with 2% charcoal-treated FCS) was not changed during the experiment. After 48 h the cAMP response to PTH (10^{-8}M), isoproterenol (10^{-8}M) and prostaglandin E_2 (PGE_2, 10^{-8}M) was determined. cAMP and DNA content of the cells was assayed as described in Methods. *$p<0.01$ versus control.

tenuation of the cAMP response to both PTH and isoproterenol (results not shown). Therefore, it was surprising that we observed a stimulatory effect on the cAMP response to isoproterenol in primary cultures of ROB cells after treatment for 2 days with 1,25-$(OH)_2D_3$, whereas no change in PGE_2 stimulated production of cAMP was found (Table II). The absence of a diminished cAMP response to PGE_2, after treatment with 1,25-$(OH)_2D_3$, could be due to the relatively short incubation period (2 days). Kubota et al (1985) showed that this effect only became apparent after 4 days of treatment with 1,25-$(OH)_2D_3$. However, this does not explain the 1,25-$(OH)_2D_3$-induced augmentation of the cAMP response to the β-adrenergic agonist, isoproterenol.

From the time course experiments (Fig. 4) it seems clear that the adenylate cyclase appears to be more highly expressed in subconfluent, rapidly growing cultures (day 2) than in confluent cultures (day 4). This is the case for both the PTH- and isoproterenol-stimulated cAMP response. A similar phenomenon was observed when we compared cultures of ROB cells seeded at low or high density (appendix paper 1). In this respect it is of interest that the 1,25-$(OH)_2D_3$-induced augmentation of the cAMP response to isoproterenol is only expressed during the long growth phase of the culture (Fig. 4, day 2). At the same time 1,25-$(OH)_2D_3$ seems to elicit the lowest attenuation of the cAMP response to PTH. Whether these observations in proliferating cells reflect rapidly dividing immature OB cells (preosteoblasts ?) and the subsequent change in confluent cultures is an expression of more mature OB cells (osteoblasts) remains to be established. The major problem to investigate this further will be the fact that primary cultures of OB cells consist, by definition, of a heterogenous cell population. Therefore, one cannot exclude differences in the effect of 1,25-$(OH)_2D_3$ on cells with a different state of maturation. On the other hand, also changes in 1,25-$(OH)_2D_3$ receptor number could play a role (see also chapter 4). However, irrespective of these considerations our results do suggest that the phase of the

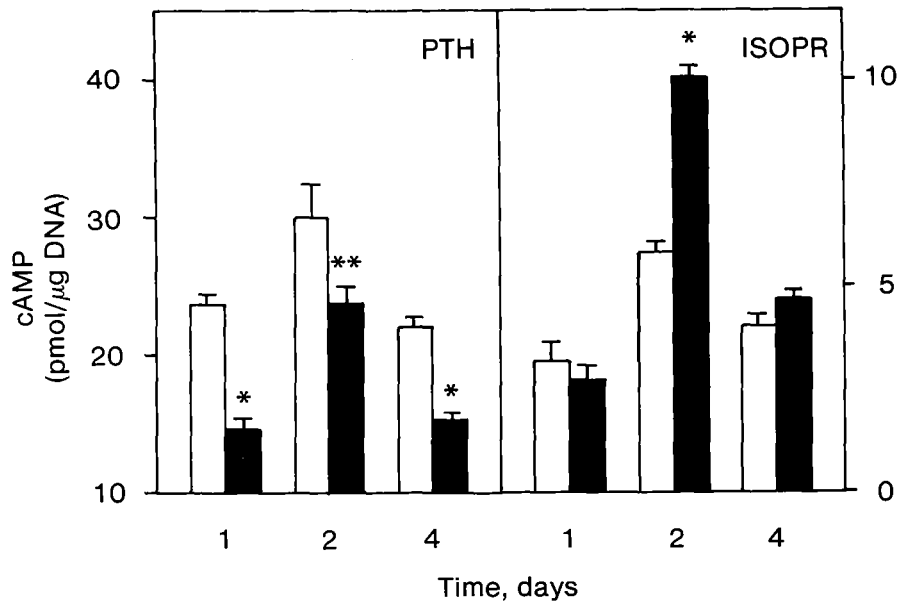

*Fig. 4: Time-course of the 1,25-$(OH)_2D_3$ effect on the PTH (10^{-8} M) and isoproterenol (ISOPR, 10^{-5} M) stimulated cAMP response. ROB cells were seeded in αMEM with 10% FCS at a density of 20.10^3 cells/cm^2. After 24 h the medium was changed to αMEM with 2% charcoal-treated FCS with or without 1,25-$(OH)_2D_3$ (10^{-8}M). The medium was changed at 48 h. cAMP was assayed as described in Methods. The results are expressed as means ± SEM of 3 experiments. Control (open bars); 1,25-$(OH)_2D_3$-treated (closed bars). *$p<0.01$; **$p<0.05$ versus control.*

cell cycle and/or cell-cell interaction may be of ciritical importance in determing the ultimate effect of 1,25-$(OH)_2D_3$ on the cAMP response to PTH and isoproterenol.

Finally, receptors for 1,25-$(OH)_2D_3$ have been reported to be present in many tissues not previously recognized as target organs for the hormone (Chapter 1, Table II). Also the adenylate cyclase complex is ubiquitous as a second messenger system. Therefore, it is possible that modulation of adenylate cyclase by 1,25-$(OH)_2D_3$ may occur in other cell types. Recent studies (Rubin and Catherwood, 1984, 1987) indicate that this is the case.

3.3.3 In vitro synthesis of 1,25-$(OH)_2D_3$ by osteoblast-like cells

It was formerly believed that 1,25-$(OH)_2D_3$ was synthesized exclusively in the kidney. Previous studies, however, showed evidence in support of extrarenal production of 1,25-$(OH)_2D_3$ as well as 24,25-$(OH)_2D_3$ in a number of tissues. Included in these

tissues are bone (Turner et al, 1980; Howard et al, 1981, 1983; Pols et al, 1985), cartilage (Garabedian et al, 1978), placenta (Tanaka et al, 1979), chorioallantoic membrane (Puzas et al, 1980) and cells of the monocyte/macrophage lineage (review Mason, 1985). Moreover, with the advent of sensitive methods for the measurement of 1,25-$(OH)_2D_3$ detectable levels of circulating 1,25-$(OH)_2D_3$ have been shown in humans after bilateral nephrectomy (Jongen et al, 1981, 1984; Lambert et al, 1981). Taken together these observations suggest that the production of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ is more widespread than previously believed.

As outlined in detail in appendix paper 2, we also found 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ production in chicken OB cells (COB cells). In our study the identification of the dihydroxyvitamin D_3 metabolites was based on comigration with authentic standards in different HPLC systems and the difference in sensitivity to periodate cleavage of the tritiated metabolites formed. Recent evidence indicates that in vitro a 25-$(OH)D_3$ metabolite can be formed, which is indistinguishable from true 1,25-$(OH)_2D_3$ with the means we used for identification (Lester et al, 1984; Cohen and Gray, 1984). This metabolite has been identified by mass spectral (m.s.) analysis as 19-nor-10-keto-25-$(OH)D_3$ (Lester et al, 1984). However, in COB cells, the OB cell culture system used in our study, the production of 1,25-$(OH)_2D_3$ has been unequivocally documented by m.s. analysis (Turner et al, 1983).

The observation that hydroxylation to 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ occurs at 10nM 25-$(OH)D_3$, a concentration even below the serum levels of 25-$(OH)D_3$ in humans and chicks (50-100 nM), indicate that the 1α- and 24-hydroxylase in COB cells can utilize relatively low substrate concentrations. Also the recently reported apparent Km values for the 1α-hydroxylase and 24-hydroxylase, 5 nM and 15 nM, respectively, support the concept that both hydroxylases are specific and not only active at pharmacological doses of 25-$(OH)D_3$ (Puzas et al, 1987).

In most in vitro systems, used for the study of 25-$(OH)D_3$ metabolism, the cells are maintained in serum-free medium without vitamin D binding protein (Turner et al, 1980; Howard et al, 1981; Puzas et al, 1987). This is a circumstance that does not occur in vivo and might influence the synthesis in OB cells of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$. Therefore, we used varying concentrations of bovine serum albumin (BSA) in the incubation medium, in an attempt to mimick the influence of vitamin D binding protein in the in vivo situation. As described in appendix paper 2 we found a striking difference in the time course of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ at different BSA concentrations. Changes in the availability of the substrate and further conversion of both dihydroxyvitamin D_3 metabolites along the C24-oxidation pathway (see also chapter 4) seems to be modulated by the actual BSA concentration. Therefore, these results strongly suggests that interpretation of in vitro data concerning vitamin D metabolism is not possible without knowledge of the effects of vitamin D binding proteins.

To study the influence of the 24-hydroxylase on 1,25-$(OH)_2D_3$ accumulation in more detail we used incubations with a low BSA concentration, because under these conditions we observed a high activity of this enzyme (appendix paper 2). As shown in Table III preincubation with 1,25-$(OH)_2D_3$ caused a diminished accumulation of 1,25-$(OH)_2D_3$ and an enhanced accumulation of 24,25-$(OH)_2D_3$ in a manner similar to that described in kidney cells (Henry, 1984). Recently, we have found that concentration as low as 10^{-11} M 1,25-$(OH)_2D_3$ are able to induce 24-hydroxylase activity in OB cells (Chapter 4, section 4.3.1). Therefore, it seems possible that the 1,25-$(OH)_2D_3$ produced in the COB cells is capable to induce its own degradation through the induction of 24-hydroxylase activity. Both the observation that 1,25-$(OH)_2D_3$ is rapidly metabolized to 1,24,25-$(OH)_3D_3$ and the finding that inhibition of 24-hydroxylase activity with cycloheximide leads to an increased 1,25-$(OH)_2D_3$ accumulation support this concept (appendix paper 2). This also raises the question whether the principal function of the 24-hydroxylase is to initiate degradation of 1,25-$(OH)_2D_3$ and, consequently, whether 25-$(OH)D_3$ is only an alternate substrate for this enzyme. That 1,25-$(OH)_2D_3$ could inhibit 24-hydroxylase when 25-$(OH)D_3$ is used as substrate is suggested by the coincubation experiments (Table III), but the degree of competition between the same concentration of radioinert 1,25-$(OH)_2D_3$ and [^{3}H]25-$(OH)D_3$ remains unclear. As already proposed by Brommage and DeLuca (1985) determination of the relative Km and maximum velocity (Vmax) values of the 24-hydroxylase for both 25-$(OH)D_3$ and 1,25-$(OH)_2D_3$, and examination of the relative rates of 24-hydroxylation of these metabolites in vivo would help to address the validity of the hypothesis that the 24-hydroxylase should, in fact, be called the 1,25-$(OH)_2D_3$-24-hydroxylase.

Looking at other possible regulatory mechanisms we could not find any significant influence of changes in the medium calcium concentration on 25-$(OH)D_3$ metabolism

Table III: Effect of preincubation and coincubation with 1,25-$(OH)_2D_3$ on 25-$(OH)D_3$. metabolism.

	Accumulation of [^{3}H]25-$(OH)D_3$ metabolites	
	1,25-$(OH)_2D_3$ (pmol)/sample)	24,25-$(OH)_2D_3$ (pmol/sample)
Control	0.16 ± 0.05	1.96 ± 0.47
Preincubation	0.05 ± 0.01*	5.49 ± 1.37*
Coincubation	0.18 ± 0.06	1.53 ± 0.49

COB cells were preincubated (2 h) or coincubated with 10 nM radioinert 1,25-$(OH)_2D_3$. Accumulation of [^{3}H]1,25-$(OH)_2D_3$ and [^{3}H]24,25-$(OH)_2D_3$ in the medium (αMEM with 0.1% BSA) was assayed after an incubation of 3 h with 10 nM [^{3}H]25-$(OH)D_3$. Results are expressed as the means ± SD of 9 cultures. *$p < 0.01$ versus control.

in COB cells (Table IV). Also PTH, which is a potent stimulator of the 1α-hydroxylase in kidney cells, did not influence the synthesis of both dihydroxyvitamin D_3 metabolites (results not shown). Our data concerning the effect of low medium calcium are in contrast to the observations of Howard et al (1981). These investigators reported a decreased 24,25-$(OH)_2D_3$ accumulation, without any change in 1,25-$(OH)_2D_3$ synthesis, after culturing human bone cells for 10-12 days at 0.6 mM calcium. Despite differences in experimental conditions (culturing 16 h vs 10-12 days at low calcium) and culture systems, it seems premature to suggest that these data, obtained in not fully characterized human bone cell cultures, indicate that calcium controls vitamin D metabolism in these cells. Both the lack of convincing evidence concerning the identification of the 25-$(OH)D_3$ metabolites formed in human bone cells and of the long-term incubations needed to observe an effect does not permit such a conclusion.

Taken together we could only identify two factors which influenced 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ production in COB cells: 1) vitamin D binding protein(s) and 2) the ability of 1,25-$(OH)_2D_3$ to induce 24-hydroxylase activity. Furthermore, we recognize that our experiments were performed in avian bone cells, which does not necessarily mean that similar results could be obtained in OB cells from other species (Chapter 4). What can be said, though, is that in the present system we have been able to measure 1,25-$(OH)_2D_3$ production, which agrees with results of other in vitro experiments, namely those utilizing renal cell culture (Henry, 1984). The fact that these renal cell experiments have been shown to have physiological relevance supports such a relevance for our experiments. Also from a clinical point of view there exists some (circumstantial) evidence to support a role for 1,25-$(OH)_2D_3$ synthesis by osteoblasts. Comparison of the changes in the osteoclast number in vitamin D deficient patients and anephric patients shows striking differences: osteoclast number is

Table IV: The influence of different medium Ca concentrations on 25-(OH)D_3 metabolism.

	Accumulation of [^{3}H]25-(OH)D_3 metabolites	
Ca concentration (mmol/l)	1,25-$(OH)_2D_3$ (pmol/sample)	24,25-$(OH)_2D_3$ (pmol/sample)
0.6	0.14 ± 0.05	1.4 ± 0.1
1.25	0.14 ± 0.10	1.4 ± 0.7
2.5	0.14 ± 0.08	1.3 ± 0.3

COB cells were incubated in medium (αMEM with 0.1% BSA) with different Ca concentrations (0.6-2.5 mM) for 16 h. [^{3}H]-(OH)D_3 (10 nM) was added during the last 3 h of the incubation. At the end of the experiment the accumulation of [^{3}H]1,25-$(OH)_2D_3$ and [^{3}H]24,25-$(OH)_2D_3$ in the medium was analysed. Values represent the means ± SD of 5 cultures.

normal or low in vitamin D deficiency, but increased in anephric patients, despite the fact that in both situations there are low levels of $1,25\text{-}(OH)_2D_3$ and high levels of serum PTH. A major variable is the level of $25\text{-}(OH)D_3$, which is low in the vitamin D deficient state and normal in the anephric patients. Therefore, it remains a possibility that in the anephric patients the osteoblasts synthesize $1,25\text{-}(OH)_2D_3$, resulting together with the elevated PTH levels in an increased number of osteoclasts (see also Chapter 1, section 1.3.3), while in the vitamin D deficient patients this mechanism could not operate through a lack of $25\text{-}(OH)D_3$.

CHAPTER 4

Regulation of vitamin D action at the cellular level

4.1 Introduction

A direct consequence of 1,25-$(OH)_2D_3$ action in several of its target tissues is the induction of the enzyme 24-hydroxylase (Chapter 1, section 1.1.4 and 1.1.5). Substantial evidence has been accumulated to suggest that the expression/induction of the 24-hydroxylase activity represents a classical steroid hormone action (review DeLuca, 1984; Henry, 1984; Haussler, 1986). In intestinal and kidney tissue it has recently been shown that 24-hydroxylation of 1,25-$(OH)_2D_3$ to 1,24,25-$(OH)_3D_3$ only forms the initial step in a more extensive C23- and C24-oxidation of the hormone (Mayer et al, 1983; Napoli et al, 1983; Jones et al, 1983, 1984)). This so-called 'side chain oxidation pathway' (Chapter 1, section 1.1.5.2) has been proposed to represent a catabolic process leading to the excretion of the molecule. This may imply that the self-induced metabolism of 1,25-$(OH)_2D_3$ regulates the concentration of the hormone at the site of action. In this chapter data are presented, suggesting that also in OB cells this C23- and C24-oxidation pathway exists and plays a role in the regulation of the intracellular levels of and, consequently, receptor occupancy by the active form of vitamin D.

Receptor regulation could be another mechanism, besides local synthesis (appendix paper 2) and catabolism of 1,25-$(OH)_2D_3$, for modulating target cell responsiveness to vitamin D. Evidence exists for homologous regulation of a number of receptor systems, both steroid and peptide (Catt et al, 1979; Clark et al, 1980; Clark et al, 1985; Waldman et al, 1984). Therefore, we have investigated whether this phenomenon also occurs after 1,25-$(OH)_2D_3$ receptor interaction. Exposure of UMR-106 cells to 1,25-$(OH)_2D_3$ causes a rapid accumulation of receptors. Furthermore, we observed an intimate relation between 1,25-$(OH)_2D_3$-induced receptor upregulation and the self-induced metabolism of the sterol.

Finally, we examined the relation between receptor abundance and the magnitude of the biological response exerted by 1,25-$(OH)_2D_3$. For this purpose we used the receptor-mediated induction of metabolism of 1,25-$(OH)_2D_3$ as a measure of reponsiveness.

4.2 Methods

Cell culture
UMR-106 cells: see appendix paper 3 and 4.

Metabolism of vitamin D_3 metabolites
The experimental conditions for the study of [26,27^{3}H]25-(OH)D_3 and [26,27^{3}H]24,25-$(OH)_2D_3$ metabolism in UMR-106 cells were comparable to those described for the study of [^{3}H]1,25-$(OH)_2D_3$ metabolism (see appendix paper 3). The only difference was that the cells were preincubated for 1-2 h with various concentrations radioinert 1,25-$(OH)_2D_3$ to induce 24-hydroxylase activity. At the end of the preincubation period the cells were incubated with Hanks medium with 2% BSA for 10 min and after an additional wash with Hanks without BSA further incubated in the original medium (αMEM, 0.1% BSA; see appendix paper 3). After 0-2 h [^{3}H]25-(OH)D_3 or [^{3}H]24,25-$(OH)_2D_3$ was added for 1 h. Afterwards medium (and cells) were extracted with diethyl ether and chromatographed.

Chromatography
Automatic analytical HPLC was performed using a modular instrument comprising a model 440 pump, a WISP automatic sample injector, and a fixed wavelength UV detector (254 nm) (all from Waters associates). Effluent was collected in 15 sec fractions.

Separations were performed using a CPtmSpher Silica column (10 x 0.3 cm, Chrompack) at the flow rate of 0.6 ml/min, or a Zorbax CN column (25 x 0.46 cm, Du Pont) at a flow rate of 1.3 ml/min. The solvent system of choice was dependent on both the column and the substrate.

1,25-$(OH)_2D_3$ metabolites:
- CPtmSpher Silica; hexane (H)/isopropanol (I)/methanol (M) = 88:10:2.
- Zorbax CN; H/I/M = 91:7:2.

25-(OH)D_3 and 24,25-$(OH)_2D_3$ metabolites:
- CPtmSpher Silica; H/I/H_2O = 91:9:0.1% (v/v).
- Zorbax CN; H/I/M = 94:5:1.

The radioactive metabolites were identified by their retention time after calibration with standards 25-(OH)D_3, 24,25-$(OH)_2D_3$, 24-oxo-25-(OH)D_3, 24-oxo-23,25-$(OH)_2D_3$, 1,25-$(OH)_2D_3$ and 1,24,25-$(OH)_3D_3$.

1,25-$(OH)_2D_3$ receptor binding assays:
1) Whole cell binding: Monolayers of cells were exposed to [^{3}H]1,25-$(OH)_2D_3$ with or without a 200-fold excess of unlabelled hormone. Specific cellular accumulation was calculated as the difference between the [^{3}H]1,25-$(OH)_2D_3$ bound to the cell in

the absence or presence of unlabelled hormone (for details see appendix paper 3 and 4).

2) Hypertonic cytosol: After extraction and sonification of the cells in hypertonic buffer, high speed supernatants (100.000 x g) were incubated with [^{3}H]1,25-$(OH)_2D_3$ with or without a 200-fold molar excess of unlabelled 1,25-$(OH)_2D_3$ (for details see appendix paper 4).

4.3 Results and discussion

4.3.1 Side chain metabolism of vitamin D in osteoblast-like cells

The induction of the enzyme 24-hydroxylase is one of the most prominent biological responses to 1,25-$(OH)_2D_3$ presently known. Despite the fact that bioresponses to 1,25-$(OH)_2D_3$ differ considerably between the separate target tissues (chapter 1, section 1.3), the 1,25-$(OH)_2D_3$ receptor-mediated induction of 24-hydroxylase activity is frequently observed. From studies with intestinal and kidney tissue (Napoli et al, 1983; Mayer et al, 1983; Jones et al, 1983, 1984) it became apparent that 24-hydroxylation of both 25-$(OH)D_3$ and 1,25-$(OH)_2D_3$ seems only the first step in a more extensive C24- and C23-oxidation of the side chain. That such a pathway also exists in OB cells was suggested for the first time by our study of 25-$(OH)D_3$ and 1,25-$(OH)_2D_3$ metabolism in chicken OB cells (appendix paper 2).

To study the side chain metabolism of vitamin D_3 metabolites by OB cells in more detail, we used the rat osteogenic sarcoma cell line UMR-106. Using parallel incubations of primary cultures of rat OB cells and UMR-106 cells with 25-$(OH)D_3$ and 1,25$(OH)_2D_3$ as substrate (Pols et al, 1985), had shown that the metabolic profiles obtained were identical. It is furthermore important to note that in both cell types side chain metabolism of 25-$(OH)D_3$ only occurs if the cells have been preincubated with 1,25-$(OH)_2D_3$. Due to these similarities we decided to use UMR-106 cells, because this offers the distinct advantage over primary OB cell cultures of unlimited propagation of a relatively homogenous cell population.

The 25-$(OH)D_3$ metabolites formed, after preincubation with 1,25-$(OH)_2D_3$, could be identified as 24,25-$(OH)_2D_3$, 24-oxo-25-$(OH)D_3$ and 24-oxo-23,25-$(OH)_2D_3$. This identification was based on co-migration with authentic standards in two different HPLC systems and on the different HPLC patterns depending on the location of the label in the side chain (Fig. 1). As could be expected, we found that especially 24-oxo-23,25-$(OH)_2D_3$ is deficient in tritium when [23,24-^{3}H]25-$(OH)D_3$ is used as substrate, but adequately labelled when [26,27-^{3}H]25-$(OH)D_3$ is employed. Although these data do indicate that next to 24,25-$(OH)_2D_3$ 24-oxo-metabolites of 25-$(OH)D_3$ are formed, conclusive evidence was ultimately obtained by Lohnes and Jones (1987). These investigators identified the putative 24-oxo-metabolites unequivocally through chemical modification with $NaBH_4$ and periodate, and mass spectral

analysis. They also obtained evidence that UMR-106 cells produce a metabolite which was tentatively identified as 24,25,26,27-tetranor-23-(OH)D_3.

That the induction of the side chain oxidation of 25-(OH)D_3 is a 1,25-$(OH)_2D_3$ dependent phenomenon is shown in Fig. 2. Already at 10^{-11}M 1,25-$(OH)_2D_3$ induction of 24-hydroxylase activity was observed. The appearance of 24-oxo-25-(OH)D_3 and 24-oxo-23,25-$(OH)_2D_3$, respectively, was found after preincubation with slightly higher 1,25-$(OH)_2D_3$ concentrations. When 24,25-$(OH)_2D_3$ was used as a substrate it became evident that also the steps beyond the 24-hydroxylation require induction with 1,25-$(OH)_2D_3$ (Fig. 2). Taken together, these data suggest that 1,25-$(OH)_2D_3$ not only induces dose dependently the enzyme 24-hydroxylase, but also a series of enzymes which convert 24,25-$(OH)_2D_3$ further to 24-oxo-25-(OH)D_3 and 24-oxo-23,25-$(OH)_2D_3$. As shown in Fig. 3 we believe that 24,25,26,27-tetranor-23-(OH)D_3 forms the logical last metabolite in this cascade of conversions, although definitive proof is not available.

For 1,25-$(OH)_2D_3$ we (appendix paper 3) and others (Lohnes and Jones, 1987) documented a similar side chain oxidation pathway (Fig. 3). In this respect it is important to emphasize that the metabolism of 1,25-$(OH)_2D_3$, in contrast to 25-(OH)D_3, is induced by itself. Some authors (Eisman et al, 1984) do not consider this metabolism of the hormone as purely catabolic, since at least one of the putative metabolites, 24-oxo-1,25-$(OH)_2D_3$, has been shown to possess high affinity for the 1,25-$(OH)_2D_3$ receptor (Napoli et al, 1983). However, the lower biological activity of both 1,24,25-$(OH)_3D_3$ and 24-oxo-1,25-$(OH)_2D_3$ compared to 1,25-$(OH)_2D_3$ (Mayer et al, 1983) does not support an important role for side chain oxidation products in the ultimate action of vitamin D.

In conclusion, we have shown an extensive side chain oxidation that is specifically induced by 1,25-$(OH)_2D_3$, but not 25-(OH)D_3. Recent reports indicate the existence of a similar 1,25-$(OH)_2D_3$-inducible pathway in kidney (Jones et al, 1983), intestine (Mayer et al, 1983), breast cancer cells (Eisman et al, 1984) and the human leukemic cell line HL-60 (Reddy et al, 1987). Therefore, we suggest that the self-induced metabolism of 1,25-$(OH)_2D_3$ may be a general mechanism for the regulation of the ultimate hormone concentration at its site of action. That this has also implications for 1,25-$(OH)_2D_3$ receptor occupancy and regulation will be discussed in the next section.

4.3.2 Some aspects of 1,25-$(OH)_2D_3$ receptor regulation

Several factors have already been identified which are capable of modulating the cellular content of 1,25-$(OH)_2D_3$ receptors (review Haussler, 1986). For instance compounds such as glucocorticoids (Hirst and Feldman, 1982; Chen et al, 1983; Manolagas et al, 1984) and retinoic acid (Petkovitsch et al, 1984; Chen and Feldman, 1985) can regulate 1,25-$(OH)_2D_3$ receptor levels. Chen and Feldman (1981) reported

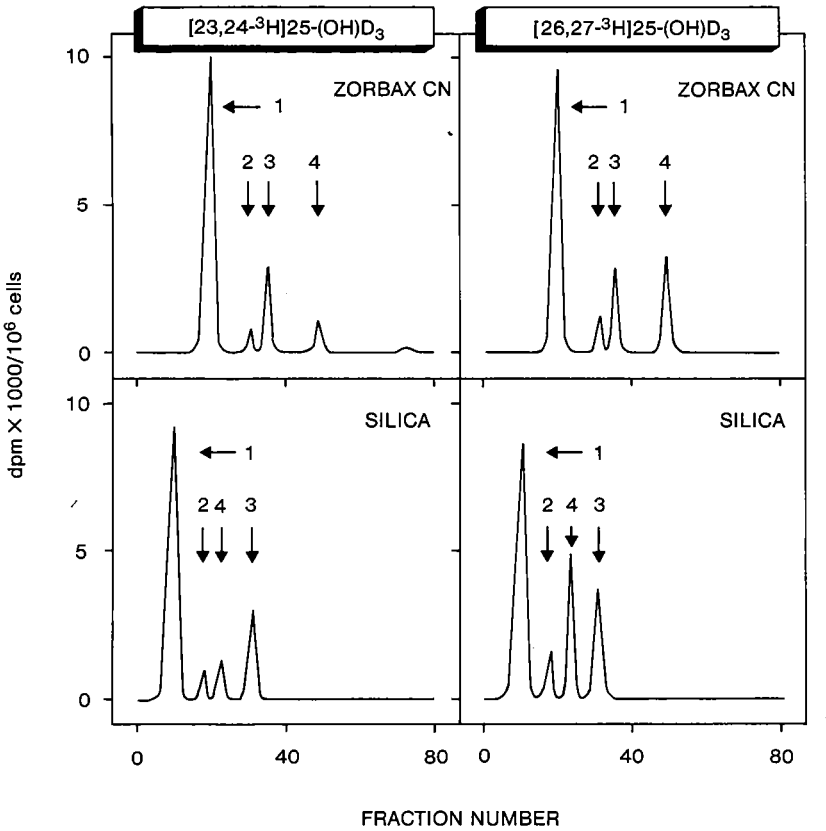

Fig. 1: HPLC analysis of [^{3}H]25-(OH)D_3 metabolites formed by UMR-106 cells. Cells were preincubated during 2 h with 0.5 nM 1,25-$(OH)_2D_3$ in αMEM medium with 0.1% BSA. Afterwards cells were washed and incubated during 1 h with 10 nM [23,24-^{3}H]- or [26,27-^{3}H]25-(OH)D_3 (left and right panels, respectively). The medium and cells were extracted together and chromatographed on a Zorbax CN column (upper panels) or a CPtmSpher Silica column (lower panels) as described in Methods. The peaks represent: 25-(OH)D_3 (1); 24-oxo-25-(OH)D_3 (2); 24,25-$(OH)_2D_3$ (3); 24-oxo-23,25-$(OH)_2D_3$ (4).

that levels of the 1,25-$(OH)_2D_3$ receptor correlate positively with the rate of cell division in primary cultures of mouse OB cells. In this section we will demonstrate that 1,25-$(OH)_2D_3$ itself can regulate the level of its own receptor in UMR-106 cells and show that this is tightly linked to the regulation by the hormone of its own metabolism. Furthermore, data will be presented indicating a modulation of receptor level by culture conditions.

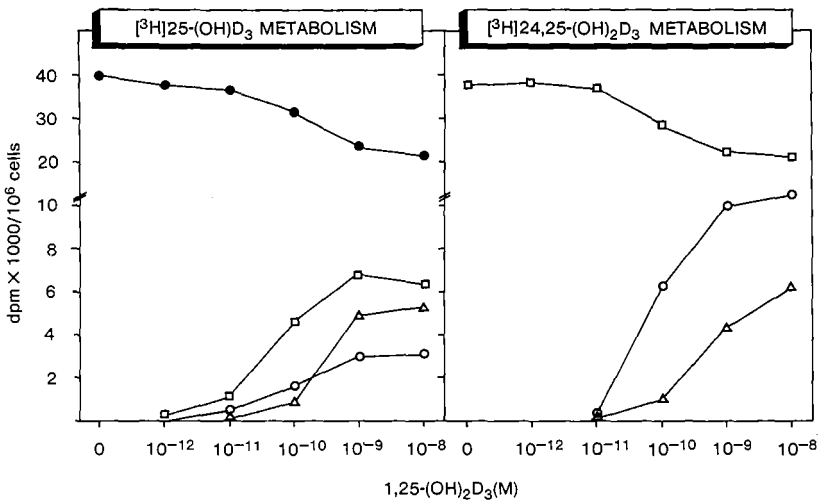

Fig. 2: The effect of 1,25-(OH)$_2$D$_3$ on the metabolism of [^{3}H]25-(OH)D$_3$ and [^{3}H]24,25-(OH)$_2$D$_3$. Cells were preincubated during 2 h with various concentrations 1,25-(OH)$_2$D$_3$. Afterwards cells were washed and additionally incubated during 1 h with 10 nM [26,27-^{3}H]25-(OH)D$_3$ (left panel) or 10 nM [26,27-^{3}H]24,25-(OH)$_2$D$_3$ (right panel). The medium and cells were extracted together and analysed by HPLC as described in Methods. (●) 25-(OH)D$_3$; (□) 24,25-(OH)$_2$D$_3$; (○) 24-oxo-25-(OH)D$_3$; (△) 24-oxo-23,25-(OH)$_2$D$_3$. Values are means of 4 cultures with SD values less than 10%.

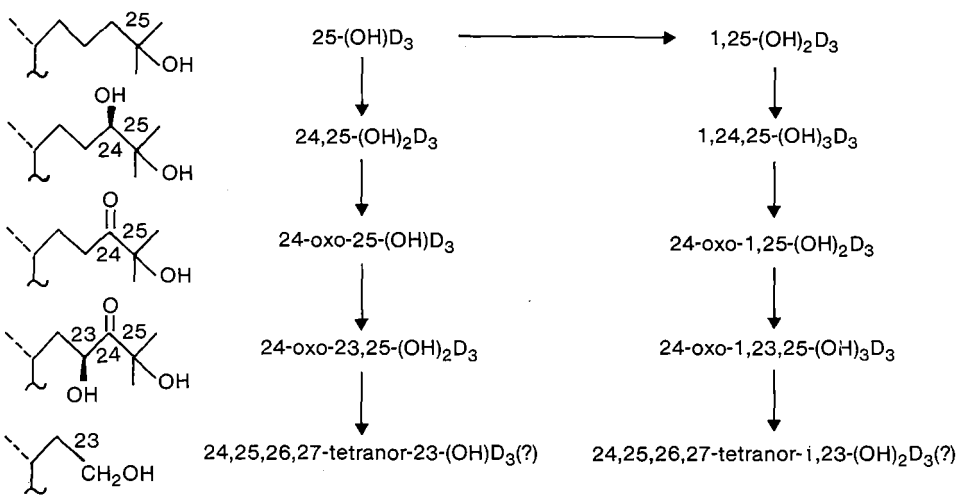

Fig. 3: The side chain oxidation pathway of 25-(OH)D$_3$ and 1,25-(OH)$_2$D$_3$.

The time-course of specific binding in UMR-106 cells, measured as specific cellular accumulation (SCA) of 1,25-$(OH)_2D_3$, exhibited two components: an ascending phase, reaching a maximum at 90-120 min, which represents the association of the hormone with its receptor, and a rapid descending phase, closely associated with the decrease of hormone concentration due to side chain oxidation (Fig. 4). To investigate receptor accumulation in the absence of metabolism we needed a specific inhibitor, which interferes with 1,25-$(OH)_2D_3$ metabolism at the level of the enzymes involved. As outlined in detail in appendix paper 3, the imidazole ketoconazole meets this requirement. If metabolism was inhibited with this compound the second phase of the time course of SCA did not show a decline, but instead a sharp increase in receptor accumulation (Fig. 4). These results indicate that the 1,25-$(OH)_2D_3$ receptor appears to undergo up-regulation. Moreover, the sensitivity of this up-regulation to actinomycin D (appendix paper 4) indicates that this receptor modulation is dependent upon new RNA synthesis.

In view of the considerable evidence that has recently accumulated (Costa et al, 1985; Mangelsdorf et al, 1987; Pan and Price, 1987) we also initially interpreted our results as an indication that the observed receptor up-regulation in UMR-106 cells represents a 1,25-$(OH)_2D_3$-mediated phenomenon (appendix paper 3). However, this became questionable after we obtained evidence that the introduction of the serum-free incubation medium, at the start of the whole-cell binding experiments, by itself induced a modulation of cellular 1,25-$(OH)_2D_3$ receptor levels. As can be seen in Fig. 5, a medium change from αMEM with 2% FCS to a serum-free medium with 0.1% BSA resulted within 3 h in a doubling of the cellular receptor content, followed by a decline to levels ultimately below the initial value. Furthermore, as found for the homologous up-regulation of 1,25-$(OH)_2D_3$ receptors (Costa et al, 1985) the initial rise of receptor level in response to the medium change was inhibited by actinomycin D. Although these findings still suggest that the elevation of binding sites, observed in our whole cell binding experiments, is dependent upon new RNA synthesis, they do not support the hypothesis that 1,25-$(OH)_2D_3$ is the only factor responsible for this phenomenon. On the contrary, a not yet identified factor related to our culture conditions could play a role.

The definitive proof for the existence of a homologous up-regulation of 1,25-$(OH)_2D_3$ receptors in UMR-106 cells was obtained from preincubation experiments with radioinert hormone and subsequent measurement of 1,25-$(OH)_2D_3$ binding in cytosolic extracts. As outlined in detail in appendix paper 4, a 4 h preincubation of the UMR-106 cells with various concentrations 1,25-$(OH)_2D_3$ resulted in a dose dependent up-regulation of its receptor. In this respect it is important to emphasize that the receptor levels measured in vehicle treated cultures of these 4 h preincubation experiments (Fig. 6) were equal to those found 4-6 h after the medium change in the previous experiment (Fig. 5). In other words, the homologous up-

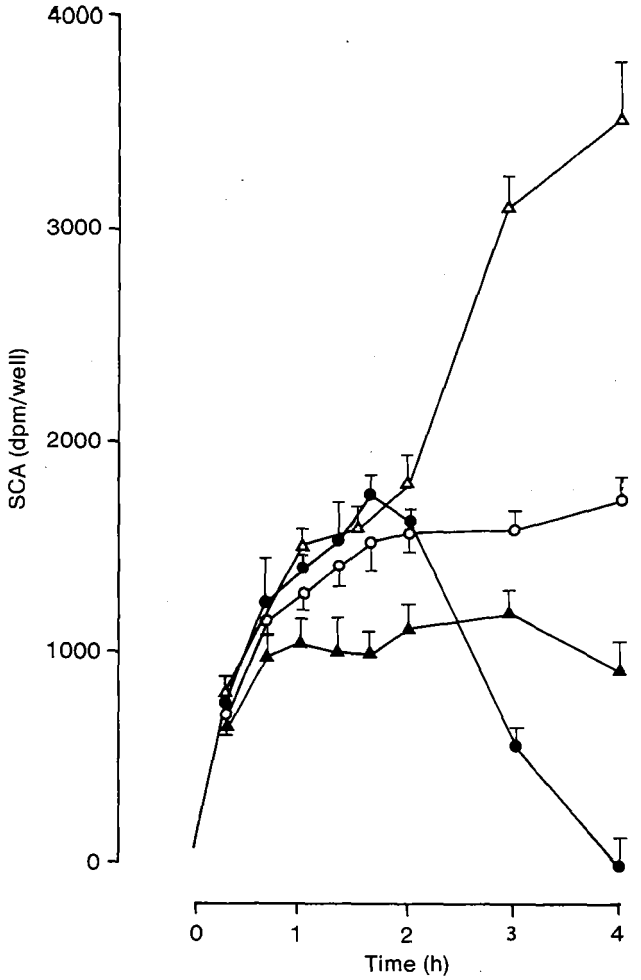

Fig. 4: A typical time course of the effect of ketoconazole, cycloheximide and actinomycin D on specific cellular accumulation (SCA) of 1,25-$(OH)_2D_3$ in UMR-106 cells. Confluent cultures (24-well dishes) were incubated with 0.5 nM [^{3}H]1,25-$(OH)_2D_3$ (with or without a 200-fold molar excess of unlabelled 1,25-$(OH)_2D_3$) alone or in the presence of ketocanazole (10^{-4}M), cycloheximide (2 ug/ml) or actinomycin D (1 ug/ml), respectively. SCA was measured at the times indicated. Each well contained approximately 10^6 cells. Values are means ± SD of 3 cultures. Control (●); ketoconazole (△); cycloheximide (▲); actinomycin D (○). From appendix paper 4.

regulation of 1,25-$(OH)_2D_3$ receptors seems to be superimposed on the up-regulation caused by the medium change at the start of the experiment. The sensitivity of the homologous receptor up-regulation to the RNA synthesis inhibitor actinomycin D

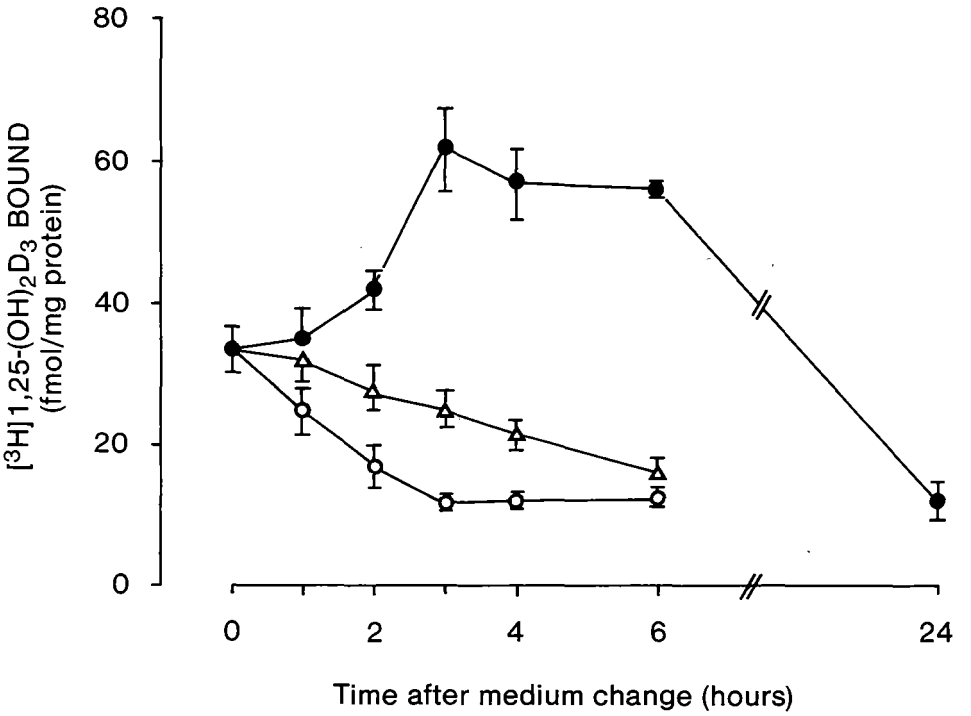

Fig. 5: Time course of receptor modulation following a culture medium change in the absence or presence of cycloheximide or actinomycin D. After the cells were cultured for 24 h in αMEM with 2% charcoal treated FCS, the medium was changed to a serum-free medium (0.1% BSA) without or with cycloheximide (10 ug/ml) or actinomycin D (1 ug/ml). Receptor content of the cells was assayed from 0 to 24 h after the medium change. Receptor levels were measured by incubation of high speed supernatants of 0.3 M KCl extracts with a saturing concentration (1 nM) of [³H]1,25-(OH)₂D₃, as described in Methods. Values are means ± SD of 3 cultures. Control (●); cycloheximide (○); actinomycin D (△).

(Fig. 6) provides support for the hypothesis that this phenomenon appears to be due to a 1,25-$(OH)_2D_3$ receptor-mediated event. Treatment of 3T6 fibroblasts with 1,25-$(OH)_2D_3$ also leads to an obvious increase in the apparent concentration of the 1,25-$(OH)_2D_3$ receptor mRNA signal (McDonnell et al, 1987). This increase qualitatively corresponds to that seen at the protein level and thus corroborates our view that 1,25-$(OH)_2D_3$ up-regulates its receptor, at least in part, via a direct increase in receptor mRNA.

Inhibition of protein synthesis with cycloheximide was used to determine turnover of receptor molecules in the presence and absence of saturating 1,25-$(OH)_2D_3$ concentrations. When cycloheximide was added to the cultures in a 1,25-$(OH)_2D_3$-

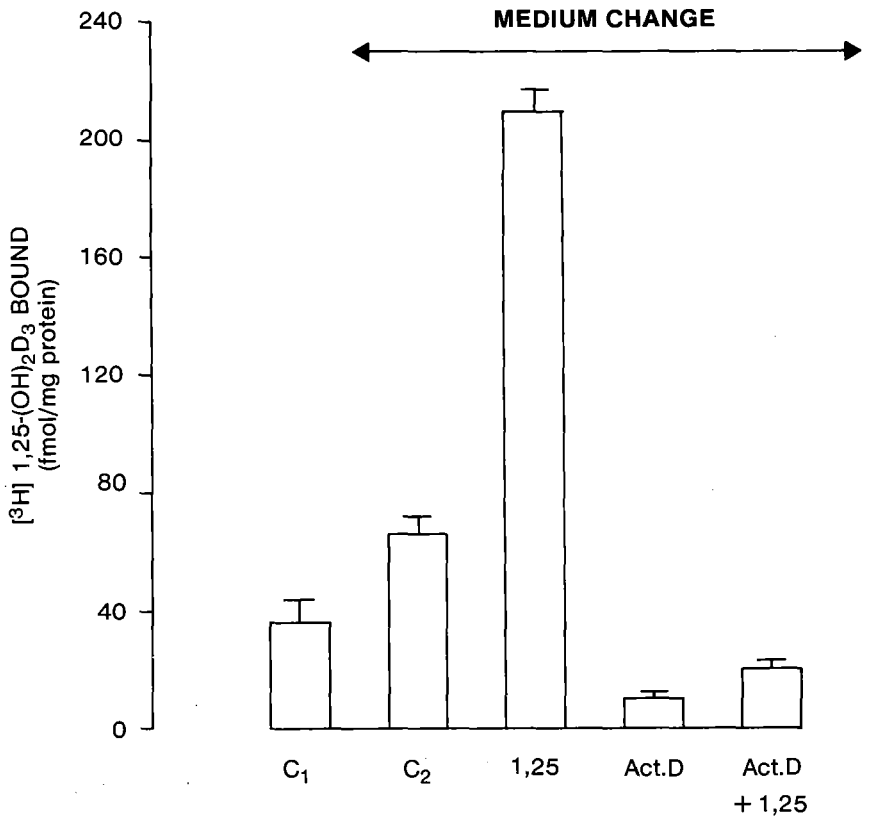

Fig. 6: The effect of actinomycin D on receptor up-regulation induced by a medium change in the absence or presence of 1,25-$(OH)_2D_3$. After the cells were cultured for 24 h in αMEM with 2% charcoal-treated FCS, the medium was changed to a serum-free medium with 0.1% BSA and the following compounds: Vehicle (C_2), 10^{-9}M 1,25-$(OH)_2D_3$ (1,25) or 1 ug/ml actinomycin D (Act. D). Receptor content of the UMR-106 cells was assayed at 0 h (C_1) and 4 h after the medium change (C_2, 1,25, Act. D and 1,25 + Act. D). Values are means ± SD of 3 cultures.

depleted medium, cellular receptor levels declined within 3 h to about 30% of the initial value (Fig. 5). The rapidity of this loss is in agreement with the short half-life of the unoccupied receptor in another osteosarcoma cell line, ROS 17.2 (Pan and Price, 1987). It also indicates that maintenance of the 1,25-$(OH)_2D_3$ receptor level is dependent on continuous de novo protein synthesis. In this respect it was not surprising that we measured a lower maximum SCA than obtained in control cultures when 1,25-$(OH)_2D_3$ and cycloheximide were added together (Fig. 4) (see also appendix paper 4). However, after the maximum SCA was reached, the further course of

specific binding did not show a tendency to descend, as would have been expected in the absence of receptor synthesis. It seems possible that this particular course of specific binding is due to an incomplete inhibition of receptor synthesis by cycloheximide, despite the high concentrations of this compound used (up to 10 ug/ml). This interpretation is supported by the finding that puromycin eliminates 1,25-$(OH)_2D_3$ binding more effectively than cycloheximide (Mangelsdorf et al, 1987; McDonnell et al, 1987). On the other hand, the absence of the expected decline of specific binding in the presence of cycloheximide may also represent a change in the degradation rate of the receptor, due to occupancy with 1,25-$(OH)_2D_3$. Also the observation that in the presence of actinomycin D the second phase of receptor accumulation (Fig. 4) did not show a decline could be compatible with a prolonged half-life of occupied receptors.

Taken together our results do indicate the existence of homologous up-regulation of 1,25-$(OH)_2D_3$ receptors in UMR-106 cells, although at present we can not clearly seperate to what extent this is caused by the ability of the hormone to enhance receptor synthesis rate and/or to decrease degradation rate. However, recently reported evidence supports our view that it seems possible that up-regulation of receptors, after exposure of cells to 1,25-$(OH)_2D_3$, results from both a prolongation of receptor half-life and an enhancement of its synthesis rate (Costa and Feldman, 1987).

As outlined in appendix paper 4, an important difference between UMR-106 cells and other cell lines used for the study of 1,25-$(OH)_2D_3$ receptor regulation (Sher et al, 1984; Pan and Price, 1987; McDonnell et al, 1987) appears to be the rapid metabolism of 1,25-$(OH)_2D_3$ in these cells. This provided us with the opportunity to study the interaction between metabolism and receptor regulation in relatively short-term incubations. From these studies (appendix paper 4) it became clear that the self-induced metabolism of 1,25-$(OH)_2D_3$ has a dual effect: 1) directly by its regulation of the cellular hormone level, and 2) indirectly by its ability to modulate the ligand-dependent regulation of the 1,25-$(OH)_2D_3$ receptor.

At first sight it may be somewhat controversial that at the same time both receptor up-regulation and metabolism are induced, because in some aspects they seem to be counteracting mechanisms. However, the association between receptor abundance and the rate of side chain metabolism (section 4.3.3) could just indicate that both mechanisms reflect a potentially flexible system for the regulation of the cellular response to 1,25-$(OH)_2D_3$. For instance, the ligand-dependent up-regulation of cellular receptor levels could provide a mechanism for enhancing the sensitivity of a target cell by amplifying the primary hormonal signal. On the other hand the higher rate of catabolism of the hormone induced at higher 1,25-$(OH)_2D_3$ receptor levels facilitates a rapid and efficient decrease of the cellular response to 1,25-$(OH)_2D_3$, including receptor up-regulation. Such a mechanism could implicate that the ability of a target cell to exhibit homologous receptor up-regulation may be dependent on the

basal receptor concentration. Support for this concept was recently presented by Costa and Feldman (1986). These investigators showed that treatment of vitamin D deficient rats with 1,25-$(OH)_2D_3$ resulted in a substantial up-regulation of 1,25-$(OH)_2D_3$ in kidney, but a marginal up-regulation of intestinal receptors. The basal levels of 1,25-$(OH)_2D_3$ receptors in this study were considerably higher in the intestine than in the kidney. Therefore, it is conceivable that the rate of induced catabolism of the hormone was higher in the intestine (Mayer et al, 1983) and, consequently, prevented a futher increment of specific binding sites.

In conclusion cellular 1,25-$(OH)_2D_3$ levels appear to be regulated in a complex manner. We and others (Costa et al, 1985, 1986; Pan and Price, 1987; McDonnell et al, 1987) have shown that the hormone regulates the level of its own receptor. The biochemical basis for the homologous up-regulation of 1,25-$(OH)_2D_3$ receptors seems a simultaneous increase of receptor synthesis and a decreased rate of receptor degradation. The self-induced metabolism of the hormone provides a mechanism that could set a limit to the ligand-dependent up-regulation at the cellular level. The modification of cellular receptor content in response to a medium change adds an additional level of complexity to the problem of receptor regulation. However, regardless of the multiple factors involved, the regulation of 1,25-$(OH)_2D_3$ receptor levels probably represents a key biochemical event in the mechanism of action of the active form of vitamin D.

4.3.3 1,25-$(OH)_2D_3$ receptor level as critical determinant of functional activity

Regulation of 1,25-$(OH)_2D_3$ receptor number could be an important mechanism modulating target cell responsiveness on the condition that changes in receptor content of the cell are associated with changes in the functional response to the hormone. Although we do not yet understand the specific basis of how receptor content of UMR-106 cells is modulated in response to a culture medium change (section 4.3.2), the finding nevertheless provides a system to explore whether there exists a relationship between cellular receptor content and the magnitude of the biological response to 1,25-$(OH)_2D_3$. Furthermore, the biphasic nature of this particular 1,25-$(OH)_2D_3$ receptor modulation offers also the oppertunity to study the functional response to the sterol at both an up- and down-regulated number of receptors (3 h versus 24 h after the medium change, Fig. 5).

One of the cell functions that has been shown to be affected by 1,25-$(OH)_2D_3$ via a receptor-mediated mechanism is the induction of the side chain oxidation of 25-$(OH)D_3$ (section 4.3.2). Therefore, this bioresponse was used as a measure of changes in cellular functional responsiveness associated with changes in receptor content. To prevent interference of homologous receptor up-regulation, preincubation with 1,25-$(OH)_2D_3$ was limited to 1 h.

The results illustrated in Fig. 7 clearly indicate that the induction by 1,25-$(OH)_2D_3$

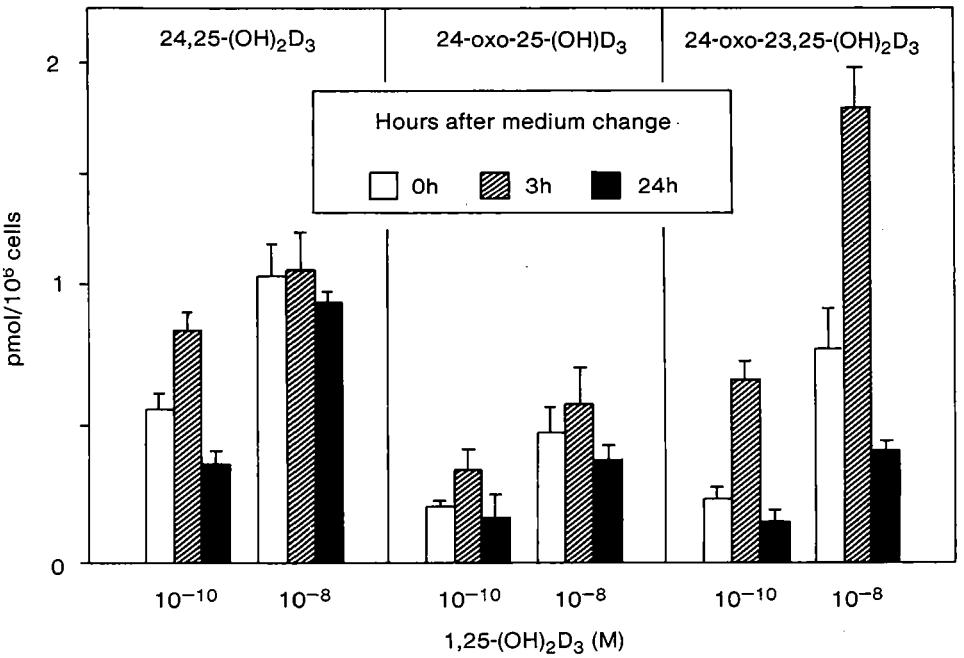

Fig. 7: Comparison of the rate of the 1,25-$(OH)_2D_3$-induced side chain metabolism in UMR-106 cells with different 1,25$(OH)_2D_3$ receptor levels. At 0, 3 and 24 h after a medium change (from αMEM with 2% FCS to αMEM with 0.1% BSA) cells were exposed to various concentrations 1,25-$(OH)_2D_3$ for 1 h. At the end of the preincubation period the cells were washed and incubated in the original medium (αMEM, 0.1% BSA). After 2 h 10 nM [^{3}H]25-$(OH)D_3$ was added for 1 h. Afterwards medium and cells were extracted together and analysed by HPLC as described in Methods. For the 1,25-$(OH)_2D_3$ receptor content of the cells at 0, 3, 24 h after the medium change, see Fig. 5. Values represent the means ± SD of 3 cultures. Left panel, 24,25-$(OH)_2D_3$; Middle panel, 24-oxo-25-$(OH)D_3$; Right panel, 24-oxo-24,25-$(OH)_2D_3$.

of C23- and C24-oxidation of the side chain was modulated by the preceding medium change and closely paralelled the changes in receptor level (Fig. 5). The apparent lack of correlation between 24,25-$(OH)_2D_3$ accumulation and receptor levels at maximally inducing 1,25-$(OH)_2D_3$ concentrations is explained by its higher rate of degradation to 24-oxo-metabolites. This observation also emphasizes the hazards associated with the determination of intermediate metabolites, like 24,25-$(OH)_2D_3$ accumulation, as the only measure of responsiveness.

The inverse correlation between the amount of substrate recovered and the number of receptors (Fig. 8) further support the hypothesis that alterations in receptor levels result in corresponding changes in the ability of 1,25-$(OH)_2D_3$ to mediate a

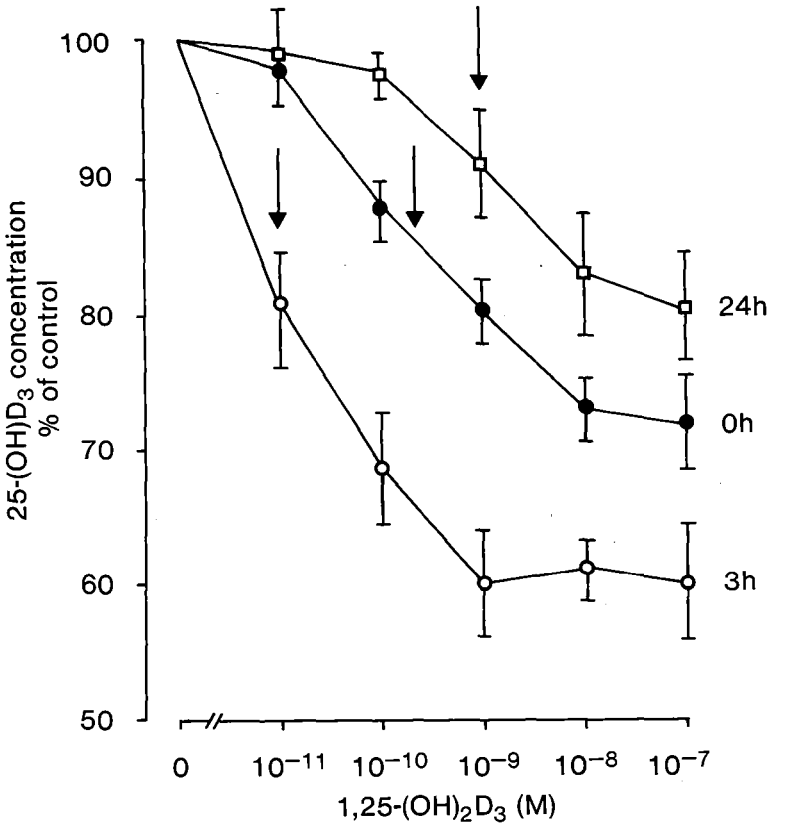

Fig. 8: Comparison of the 1,25-$(OH)_2D_3$-induced loss of [^{3}H]25-$(OH)D_3$ substrate concentration in cells with different 1,25-$(OH)_2D_3$ receptor levels. Experimental conditions were the same as described in the legend to Fig. 7. (●) 0 h, (○) 3 h and (□) 24 h after the medium change. The values represent the means ± SD of three cultures. For each of the three conditions studied, the apparent ED_{50} is indicated with an arrow.

bioresponse as measured by the induction of side chain metabolism. In addition, the apparent ED_{50} for the induction of metabolism, calculated from the 1,25-$(OH)_2D_3$ concentration-dependent decrease of the remaining 25-$(OH)_2D_3$ substrate concentration (Fig. 8), also indicates that this ED_{50} for high receptor cells (3 h) was lower than that of control cultures (0 h) and cells with a down-regulated number of receptors (24 h). Chen et al (1986) reported similar increases in the magnitude and sensitivity of other bioresponses to 1,25-$(OH)_2D_3$ (osteocalcin production and collagen synthesis), if 1,25-$(OH)_2D_3$ receptor content of primary rat OB cell cultures was up-regulated by pretreatment of the cells with glucocorticoids.

Although the increased sensitivity of the response to 1,25-$(OH)_2D_3$ could be explained by a change in receptor affinity for the hormone, we did not observe a significant change of the apparent K_d of the receptor at the different time-points after the medium change (results not shown). Also other investigators could not find a difference in the affinity of 1,25-$(OH)_2D_3$ for its receptor when the cellular receptor was up- or down-regulated by means of a medium change (Hirst and Feldsman, 1983) or by compounds such as glucocorticoids (Manolagas et al, 1984; Hirst and Feldman, 1982; Chen et al, 1986) and retinoic acid (Petkovich et al, 1984; Chen and Feldman, 1985). However, these data do not exclude the possibility that the DNA-binding properties of the up- and down-regulated receptors have been changed. Furthermore, the relatively short preincubation with 1,25-$(OH)_2D_3$ (1 h) clearly represents non-equilibrium conditions, especially at low receptor and low 1,25-$(OH)_2D_3$ levels. It is evident that further investigation will be necessary to elucidate the mechanism(s) responsible for the increased sensitivity of the response to 1,25-$(OH)_2D_3$ at higher cellular content of receptors.

In conclusion, our data suggest that receptor up-regulation increases the magnitude and sensitivity of the response to 1,25-$(OH)_2D_3$, which support the hypothesis that 1,25-$(OH)_2D_3$ receptor regulation could be a potent mechanism for modulating the action of the active hormonal form of vitamin D at the cellular level. However, it would be premature to extrapolate these in vitro findings to the in vivo situation.

CHAPTER 5

Patterns of reaction to treatment of predialysis renal osteodystrophy with 1α-hydroxyvitamin D_3

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5.1 Introduction

Since the discovery of 1,25-$(OH)_2D_3$ as the hormonally active form of vitamin D the mechanism of its skeletal effects has been extensively studied (Raisz and Kream, 1983; Puzas et al, 1984; Haussler, 1986). Recent in vitro data suggest that 1,25-$(OH)_2D_3$ may induce cell differentiation (Wong et al, 1977; Pols et al, 1986; Suda et al, 1986) and, consequently, could play a role in the regulation of the bone remodelling cells (Haussler, 1986). Nevertheless it is still not clear whether 1,25-$(OH)_2D_3$ directly promotes bone mineralization or does so through augmentation of serum calcium and phosphorus levels.

Due to the low levels of 1,25-$(OH)_2D_3$ in patients with chronic renal failure (Juttmann et al, 1981) these patients have become a model to study the effect of the active hormonal form of vitamin D. However, it has been clearly shown that subnormal availability of 1,25-$(OH)_2D_3$ is only one of the determinants of uremic bone disease. The histology of bone not only shows signs of disturbed mineralization, but in most cases also the signs of (secondary) hyperparathyroidism (osteitis fibrosa) (Brickman et al, 1974; Nielsen et al, 1980; Ritz et al, 1987). In several studies, treatment with 1α-$(OH)D_3$ or 1,25-$(OH)_2D_3$ resulted in a decrease of the features related to secondary hyperparathyroidism, such as increased bone resorption and peritrabecular fibrosis (Cushner and Adams, 1986; Juttmann et al, 1983).

Osteomalacia of renal failure seems to be due to different pathogenic processes. Using histodynamic techniques Evans et al (1982) distinguished classic osteomalacia (wide osteoid seams, low mineral appositional rates and wide, merging tetracycline labels) from a second form exhibiting reduced mineralization surfaces but normal matrix and mineral appositional rates. Especially in patients on maintenance dialysis the former type may be related to aluminum intoxication (Cournot-Witmer et al, 1981; Andress et al, 1986; Ritz et al, 1987). In this respect the observations of Peacock et al (1979) are of interest. These investigators noted improvement of

hyperosteoidosis upon administration of 1,25-$(OH)_2D_3$ to uremic patients prior to dialysis, but not in patients already undergoing hemodialysis. This could indicate the presence of a primary 1,25-$(OH)_2D_3$ deficiency dependent process in predialysis patients while upon starting chronic hemodialysis the process of matrix mineralization could become additionally disturbed by aluminum intoxication and other factors.

Unfortunately, most studies examining the effects of treatment with active vitamin D compounds on renal osteodystrophy were performed in patients on hemodialysis rendering interpretation of the data difficult, because aluminum status generally was not evaluated. Therefore, we decided to examine the effect of a relatively short-term treatment with 1α-$(OH)D_3$ on bone turnover and mineralization in predialysis patients. In this way substantial interference by aluminum intoxication was avoided. The aim of the study was to try to evaluate separately the effect of 1,25-$(OH)_2D_3$ on the two components of renal osteodystrophy, namely the mineralization disturbance and the secondary hyperparathyroidism.

5.2 Patients and methods

5.2.1 Patients and treatment

Twenty-nine patients (18 men) with chronic renal insufficiency (CRI), who were not on dialysis treatment, were studied. The creatinine clearance values and other biochemical data are given in Table I. The average age ($\pm$ S.D.) was 46.3 $\pm$ 11.0 years. The daily protein intake was restricted to between 40 and 60 g. To subsequently keep the serum phosphate (P) level at or below 1.50 mM all patients were treated with aluminum hydroxyde 1 to 2 g daily. The daily dietary Ca-intake varied from 265 to 950 mg. In the patients who had an intake below 500 mg a daily suppletion of 0.5 g of elemental Ca was given as an effervescent tablet (Ca-Sandoz®) 3 months before the study started.

Symptoms and signs of renal osteodystrophy, such as bone pain, bone deformation, (spontaneous) fractures or radiological signs of osteomalacia or hyperparathyroidism were absent.

The initial daily dose of 1α-hydroxyvitamin D_3 (1α-$(OH)D_3$; Etalpha®, LEO Pharmaceuticals) was 0.25-0.50 μg. When necessary the dosage was adjusted according to the serum Ca and creatinine levels. The average daily dose was 0.55 $\pm$ 0.11 μg.

5.2.2 Laboratory methods

Serum Ca, P, albumin, alkaline phosphatase, immunoreactive parathyroid hormone (iPTH) were determined every 4 weeks: Ca, P, albumin and alkaline phosphatase by Technicon auto-analyzer, iPTH by a two-step radioimmunoassay (RIA) for the intact hormone (iPTH (1-84)) (Hackeng et al, 1986).

In 27 patients a bone biopsy was taken before (T_0) and after 3 months of treatment

(T_3). This was done vertically from the iliac crest 3 cm dorsally from the anterior superior iliac spine with a 6 mm (internal diameter) trephine (Sacker-Nordin). For labelling dimethylchlortetracycline (Ledermycin®) 300 mg b.d. was given orally on the 20th, 19th, 6th and 5th day before taking the biopsy. Fixation, dehydration and embedding of the undecalcified bone were performed according to a modification of Burckhardt's method (Te Velde et al, 1977). With a Jung K microtome 5 μm sections were cut. Staining and the static and dynamic histomorphometric methods and the computer program have been described previously (Birkenhäger-Frenkel et al, 1977, 1980; Clermonts and Birkenhäger-Frenkel, 1985). Histomorphometry was limited to the cancellous part of the biopsy.

The distance between the tetracycline labels was measured semi-automatically with the Videoplan apparatus in sections 5 μm thick (magnification 312.5x), while the percentages of doubly and singly labelled surface were measured in adjacent 5 μm sections with a Merz grid at a magnification of 125x (Birkenhäger-Frenkel and Birkenhäger, 1987).

Thionine stain. To improve discrimination between osteoblasts (i.e. cells capable of both matrix synthesis and mineralization) and other cells covering trabecular surface (capable of mineralizing bone matrix), we used a modification of the stain Roque et al (1965) developed to demonstrate nucleic acids. We had observed that, when omitting methyl green from the staining solution using a citrate buffered thionine solution only (0.0165 g of thionine in 100 ml of 0.02 M citrate buffer, pH 5.8; staining at 40°C for 30 minutes), RNA in the cytoplasm was stained much more clearly than in the presence of methyl green. Furthermore, the mineralization front appears as a zone of very fine granules. The latter is probably due to the property of thionine to stain acid polysaccharides. Cement lines also show up clearly (Birkenhäger-Frenkel and Derkx, to be published).

In none of the biopsies aluminum could be demonstrated by means of the Malloney stain (1982).

The following parameters were measured or calculated (symbols and dimensions according to Parfitt et al (1987)):

Trabecular bone volume	:	B.Ar (%)
Osteoblast seams length	:	Ob.Pm (%)
Osteoid seams length	:	O.Pm (%)
Relative osteoid volume	:	O.Ar/B.Ar (%)
Osteoid seams width	:	O.Wi (μm)
Osteoclast number	:	N.Oc (N/mm^2)
Fibrosis area per marrow area	:	Fb.Ar/Ma.Ar (%)
(Thionine) mineralization front (underneath all osteoid)	:	M.Bd/O.Pm (%)

(Thionine) mineralization front
(underneath osteoblasts) : Ob.M.Bd/Ob.Pm (%)
Singly tetracycline labelled perimeter : sL.Pm (%)
Doubly tetracycline labelled perimeter : dL.Pm (%)
Mineral apposition rate : MAR (μm/d)

As the histologic picture in the group of CRI patients varied widely, especially with respect to the cellular parameters and fibrosis, it was decided to present the data after having divided the population into a group of 14 with an Ob.Pm below 4% (group I) and 13 with an Ob.Pm above 4% (group II).

Statistical analysis of the biochemical and histomorphometrical data (comparing T_3 with T_0) was carried out by Wilcoxon's matched-pairs signed-ranks test (two-sided). Correlations between the biochemical and histomorphometrical parameters as well as among the biochemical and among the histomorphometric parameters were evaluated by Spearman's rank correlation test (two-sided), while the comparison between parameter values in group I and II was performed by means of Mann-Whitney's U-test.

5.3 Results

Using a cut-off point of an Ob.Pm of 4 per cent we obtained a group of 14 patients with the histologic signs of a low bone turnover, but still elevated osteoid parameter values (Ob.Pm < 4, group I), while the remaining 13 patients showed the signs of hyperparathyroidism (Ob.Pm > 4, group II).

5.3.1 Biochemistry (Table I and II):
In the whole group of 29 patients creatinine clearance, serum alkaline phosphatase and intact iPTH fell and serum Ca and P rose significantly in the 3 months of observation (Table I). Initially, creatinine clearance and iPTH in group II were significant-

Table I: Biochemical data (average ± S.E.M.) of 29 patients with CRI before (T_0) and after 3 months treatment with 1α-(OH)D_3 (T_3).

	T_0	T_3	p	Normal range
creatinine clearance (ml/min)	17.6 ± 1.7	15.6 ± 1.7	< 0.001	80 – 120
Ca (mM)	2.28 ± 0.04	2.34 ± 0.03	< 0.05	2.25 – 2.65
P (mM)	1.37 ± 0.06	1.47 ± 0.07	= 0.05	0.80 – 1.50
Alkaline phosphatase (U/l)	55 ± 4.8	49 ± 3.90	< 0.05	< 45
iPTH (1-84) (pM)°	0.55 ± 0.10	0.37 ± 0.06	< 0.005	< 0.75

°n = 28

ly lower and higher, respectively, than in group I (Table II). Serum Ca, P and alkaline phosphatase did not differ between the two groups. Treatment with 1α-$(OH)D_3$ resulted in a significant decrease of the iPTH level in both groups, while serum Ca rose in group II only. Before as well as after treatment no correlation was found between serum Ca and the corresponding iPTH values. The changes in these two parameters did not correlate either.

5.3.2 Static histomorphometry (Table III):
As could be expected from the higher iPTH levels in group II we observed – besides the significantly higher Ob.Pm, which served as the dividing criterium – a higher osteoclast number and degree of fibrosis as well as higher values for the osteoid seams and volume in that group. After treatment a decline of Ob.Pm and the osteoid seams width (O.Wi) was seen in group II only.

The (thionine) mineralization front as a percentage of the osteoid seams length (M.Bd/O.Pm) was the same (about 70%) in both groups and upon treatment it rose by 10-20% in both groups. Measured underneath the osteoblast seams (Ob.M.Bd/Ob.Pm) only, the front percentage values (also about 70%) again did not differ between the two groups. With treatment it showed an increase in group II only (Table III).

As shown in Table IV, serum Ca was negatively correlated to the osteoid seams length in group II (untreated) and positively to the mineralization front (M.Bd/O.Pm) in both groups (untreated). Between Ob.Pm and the number of osteoclasts (N.Oc) no relationship was observed in group I either before or after treatment. In group II, these two parameters were not related before, but positively – with a rather high significance – after treatment ($r = 0.29$ and 0.72, respectively). The same applied to the relationship between iPTH and fibrosis (Table IV).

5.3.3 Dynamic histomophometry (Table III):
The percentage single tetracycline label (sL.Pm) was significantly higher in group II than in group I. It did not change with treatment. This pattern resembled that of O.Pm in both groups. Accordingly, before and after treatment sL.Pm and O.Pm were positively correlated (all patients) (Table IV). Double tetracycline label (dL.Pm) in group II exceeded that in group I, albeit not significantly. With treatment it showed a striking fall (by 44%) in group II only. In both groups the mineral appositional rate (MAR) was unaltered after treatment.

For a sample of patients from both groups a positive correlation was found between the mineralization front as a fraction of the total trabecular perimeter and the extent of the single tetracycline label both before and after treatment (Table IV). This applied also to group II separately, whereof 13 patients could be studied before and 12 after treatment.

Table II: Biochemical data (average ± S.E.M.) before (T_0) and after 3 months treatment with 1α-(OH)D_3 (T_3) of 14 patients with Ob.Pm < 4% (group I) and 13 patients with Ob.Pm > 4% (group II).

	Group I				Group II		
	p*	T_0	T_3	p°	T_0	T_3	p°
Creatinine clearance (ml/min)	<0.05	22.2 ±2.8	20.4 ±2.9	<0.05	13.2 ±1.3	11.1 ±1.1	<0.01
Ca (mM)	n.s.	2.36±0.06	2.40±0.04	n.s.	2.21±0.06	2.28±0.04	<0.05
P (mM)	n.s.	1.28±0.08	1.38±0.07	n.s.	1.45±0.09	1.55±0.11	n.s.
alkaline phosphatase (U/l)	n.s.	51.5 ±6.9	46.7 ±5.3	n.s.	57.9 ±7.2	50.7 ±6.0	n.s.
iPTH (1-84) (pM)	<0.005	0.33±0.06	0.22±0.06	<0.05	0.74±0.11	0.50±0.10	<0.05

p* Diff. between T_0 of group I and T_0 of group II: Mann-Whitney U-test
p° Diff. between T_0 and T_3 of each group. Wilcoxon's matched-pairs signed-ranks test
$^{+}n = 13$

Table III: Histomorphometric data (average ± S.E.M.) before (T_0) and after 3 months treatment with 1α-(OH)D_3 (T_3) of 14 patients with Ob.Pm < 4% (group I) and 13 patients with Ob.Pm > 4% (group II).

	Group I				Group II			
	p*	T_0	T_3	p°	T_0	T_3	p°
B.Ar	n.s.	20.6 ± 1.4	20.5 ± 1.0	n.s.	22.6 ± 1.1	22.6 ± 1.3	n.s.
Ob.Pm	= 0.0001	1.89 ± 0.32	1.76 ± 0.31	n.s.	9.34 ± 1.51	7.68 ± 1.53	< 0.01
O.Pm	< 0.001	31.8 ± 5.27	31.4 ± 5.29	n.s.	61.9 ± 3.9	60.9 ± 3.7	n.s.
O.Ar/B.Ar	< 0.001	5.14 ± 0.68	4.76 ± 0.69	n.s.	10.73 ± 1.01	9.91 ± 0.99	n.s.
O.Wi	n.s.	11.90 ± 1.21	11.59 ± 0.78	n.s.	13.34 ± 1.07	11.62 ± 0.93	< 0.05
N.Oc	< 0.01	0.64 ± 0.10	0.58 ± 0.17	n.s.	1.47 ± 0.26	1.21 ± 0.30	n.s.
Fb.Ar/Ma.Ar	< 0.0005	0.067 ± 0.02	0.051 ± 0.020	n.s.	0.969 ± 0.31	0.866 ± 0.300	n.s.
M.Bd/O.Pm	n.s.	*71.1 ± 2.23	77.9 ± 2.14	< 0.001	67.3 ± 2.14	79.3 ± 1.53	< 0.001
Ob.M.Bd/Ob.Pm	n.s.	*70.8 ± 4.4	78.6 ± 3.18	n.s.	65.0 ± 4.0	74.5 ± 4.4	< 0.05
sL.Pm	= 0.005	⁺8.4 ± 1.7	6.6 ± 1.3	n.s.	17.7 ± 2.0	16.7 ± 0.50	n.s.
dL.Pm	n.s.	** 7.01 ± 1.93	5.06 ± 1.77	n.s.	°°10.57 ± 2.74	4.89 ± 1.36	< 0.05
MAR	< 0.05	** 0.84 ± 0.06	0.75 ± 0.07	n.s.	°° 0.68 ± 0.03	0.73 ± 0.04	n.s.

p* Diff. between T_0 of group I and T_0 of group II: Mann-Whitney U-test
p° Diff. between T_0 and T_3 of each group: Wilcoxon's matched-pairs signed-ranks test
*n = 12; ⁺n = 9; **n = 7; °°n = 8

In most T_0 biopsies, especially in group II, many osteoblasts had poorly staining cytoplasm with the thionine stain. Others had the swollen aspect of osteoblasts producing woven osteoid. In most of the second biopsies (T_3) an improvement of osteoblast quality could be observed, with a deeper staining of the cytoplasm.

5.4 Discussion

In this study we used an osteoblast seams percentage of under and over 4 per cent as a dividing criterium, by which we obtained two groups, one with a low and another with a high bone turnover. In the patient group with an Ob.Pm exceeding 4 per cent (group II) several other characteristics also pointed to the presence of hyperparathyroidism. These characteristics comprise a higher iPTH level, a higher number of osteoclasts, a much greater area of fibrosis and a greater extent of double tetracycline label. Only the difference in the last mentioned parameter did not reach significance. Together with iPTH and Ob.Pm the extent of double label was significantly reduced by the treatment with 1α-$(OH)D_3$. Contrastingly, of the osteoid parameters only the osteoid width (O.Wi) showed - in group II - a slight decrease (see below). On the other hand, in group I (the low bone turnover group) the histologic picture had some similarity with type II osteomalacia as described by Evans et al (1982). It showed the combination of low Ob.Pm and high osteoid parameter values,

Table IV: Correlations between biochemical and histomorphometric and among histomorphometric parameters in 14 patients with Ob.Pm<4% (group I) and 13 patients with Ob.Pm>4% (group II).

	Group I				*Group II*			
parameters	T_0	p	T_3	p	T_0	p	T_3	p
Ca/O.Pm	−0.17	n.s.	−0.36	n.s.	−0.57	<0.05	−0.37	n.s.
Ca/(M.Bd/O.Pm)	0.64	<0.05	0.66	<0.05	0.58	<0.05	0.44	n.s.
PTH/(Fb.Ar/Ma.Ar)	0.48	n.s.	0.41	n.s.	0.32	n.s.	0.76	<0.01
Ob.Pm/N.Oc	0.47	n.s.	0.48	n.s.	0.29	n.s.	0.72	<0.01

	All patients			
	T_0	p	T_3	p
Ca/O.Pm	−0.43	<0.05	−0.59	<0.01
Ca/(M.Bd/O.Pm)	0.60	<0.01	0.53	<0.02
M.Bd/(sL.Pm)°	0.70	<0.001	0.63	<0.001
O.Pm/sL.Pm°	0.70	<0.001	0.69	<0.001

°n = 21

while group I was also characterized by a more moderate degree of renal failure. Furthermore, in group I the average iPTH level was in the lower half of the normal range and decreased marginally during treatment, while here Ob.Pm and dL.Pm did not respond to treatment. Therefore, we conclude that secondary hyperparathyroidism existed in group II and that it was partly suppressed by the treatment with 1α-$(OH)D_3$. Apparently the bone matrix production was reduced through a decline of the number of Basic Multicellular Units (BMU) and not through a fall of the (bone matrix and) mineral appositional rate (MAR) of those BMU's that were left. In this connection it has to be stressed that double tetracycline label can only be produced by apposition of bone matrix and its subsequent mineralization. This implies that single label does not necessarily represent exclusively BMU's just started or finished (1983), but probably also mineralization without matrix apposition.

Although the initial serum 1,25-$(OH)_2D_3$ levels, determined in a limited number of patients, were not different in the two groups (data not shown), we observed a tendency of serum Ca to be lower and serum P to be higher in group II than in group I. During treatment serum Ca rose in group II only. Therefore, it is possible that the suppression of hyperparathyroidism by 1α-$(OH)D_3$ is exerted through this moderate rise of serum Ca. However, the lack of correlation between serum Ca and iPTH does not support this. Alternatively, a direct inhibition of PTH secretion by 1,25-$(OH)_2D_3$ may be involved. Several in vivo and in vitro data are in favour of such a mechanism (Cantley et al, 1985; Slatopolsky et al, 1984).

While O.Pm, O.Ar/B.Ar and the single tetracycline label (sL.Pm) in group II were about double the values in group I, in both groups the (thionine) mineralization front occupied about 70 per cent of the osteoid seams whether covered by seams of cubic osteoblasts or not. The osteoid parameters mentioned and sL.Pm did not change upon treatment with 1α-$(OH)D_3$. Only the O.Wi showed a slight decrease in group II. The extent of the mineralization front, however, rose in both groups. In group II this concerned the total mineralization front as well as that underneath recognizable osteoblast seams. Therefore, we suggest that the mineralization of preexisting osteoid may have improved somewhat during treatment in both groups. This improvement does not appear to be dependent upon osteoblastic activity in so far we can judge cellular activity from numerical histologic data. Apparently osteoid, that has accumulated in renal osteodystrophy over long periods of time, has great difficulty in mineralizing even when treatment with for instance 1α-$(OH)D_3$ is instituted. In this respect it is of interest that in patients with predialysis CRI treated with 1α-$(OH)D_3$ for 6 months and longer O.Pm hardly came down to less than 40 per cent (Juttmann et al, 1983). The fact that in both patient groups sL.Pm did not change during treatment, while dL.Pm was halved in group II (see above) is reason to believe that in this situation single label is a marker not only of starting or finishing BMU, but also of mineralization without recent matrix apposition. In this connec-

tion it may be important that the length of the (thionine) mineralization front in our material always largely exceeded the length of the single label, while in the whole population studied both were correlated positively before and after treatment.

As serum Ca and O.Pm were negatively and serum Ca and the mineralization front (M.Bd/O.Pm) positively correlated before and after treatment in the whole group of 27 patients, the treatment may have promoted the defective mineralization by the slight increase of the serum Ca concentration. In non-dialyzed patients with CRI other investigators (Nielsen et al, 1980) found also a negative correlation between serum Ca and osteoid seams length. Balsan and co-workers (1986) showed that chronic intravenous administration of Ca led to complete healing of vitamin D-resistant rickets type II, which implies that in the absence of a receptor-effector system for $1,25\text{-}(OH)_2D_3$ Ca is able to induce sufficient mineralization of the bone matrix.

An interesting relationship that developed upon treatment in group II is that between iPTH and fibrosis. Apparently, fibrosis had accumulated in the untreated state in this group of patients. It may be disappearing under the influence of treatment, while it is replaced by new fibrous tissue depending upon the height of the PTH level.

In group I osteoblast seams length and osteoclast number had no relationship either before or after treatment. In group II a highly significant positive correlation between these parameters had developed after treatment, which suggests that the ability of the osteoblasts to transmit the PTH signal to osteoclasts has been restored by $1,25\text{-}(OH)_2D_3$. Compatible with this mechanism is also the fact that in our patients after three months treatment with $1\alpha\text{-}(OH)D_3$ Ob.Pm was reduced while N.Oc was not (yet). A similar phenomenon has been described by Bordier et al (1978) after short-term treatment of vitamin D deficient osteomalacia with $1,25\text{-}(OH)_2D_3$. This effect of $1,25\text{-}(OH)_2D_3$ may be a direct one or may depend upon the ambient Ca and/or P concentration(s). The lack of relationship between Ob.Pm and N.Oc persisting after treatment in group I may be caused by a requirement of a minimum PTH concentration for the osteoclast recruitment to occur.

In conclusion, this study indicates that treatment with $1\alpha\text{-}(OH)D_3$ suppresses secondary hyperparathyroidism and results in a moderate increase of mineralization. Furthermore, the study provides an indication that in progressive renal failure there is an inhibition of transmission of the PTH signal by the osteoblast to the osteoclast and that this phenomenon is restored by $1,25\text{-}(OH)_2D_3$.

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SECTION II

APPENDIX PAPERS

APPENDIX PAPER 1

The effects of 1,25-dihydroxyvitamin D_3 on growth, alkaline phosphatase and adenylate cyclase of rat osteoblast-like cells

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(Received 19 March 1986)
(Accepted 27 July 1986)

Summary

$1,25(OH)_2D_3$ induced a dose-dependent increase of alkaline phosphatase activity in primary cultures of fetal rat osteoblast-like cells. A slight decrease in final DNA content was found in densely seeded cultures and at 10^{-8} M $1,25(OH)_2D_3$ only. Furthermore, $1,25(OH)_2D_3$ treatment resulted in an attenuation of the cAMP response to PTH without an effect on the EC_{50} of PTH. On the other hand no change of the cAMP response to forskolin was observed. Forskolin partly restored the decline in the PTH-stimulated adenylate cyclase activity. The results indicate that physiological concentrations of $1,25(OH)_2D_3$ have a stimulatory effect on the alkaline phosphatase of osteoblast-like cells and decrease the responsiveness of these cultures to PTH. We propose that the site of action of $1,25(OH)_2D_3$ in the adenylate cyclase system is either at the level of the regulatory unit or on the interaction of the regulatory with the catalytic unit.

Key words: *Adenylate cyclase — Alkaline phosphatase — Cell proliferation — 1,25-Dihydroxyvitamin D_3 — Osteoblast-like cells — Parathyroid hormone*

Introduction

There is growing evidence that $1,25(OH)_2D_3$ not only exerts its effects on Ca and bone metabolism through stimulation of the intestinal Ca absorption, but that it

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** Abbreviations: 25-hydroxyvitamin D_3, $25(OH)D_3$; 1,25-hydroxyvitamin D_3, $1,25(OH)_2D_3$; 24,25-dihydroxyvitamin D_3, $24,25(OH)_2D_3$; parathyroid hormone, PTH; half maximally effective concentration, EC_{50}; fetal rat osteoblast-like cells, ROB cells; alkaline phosphatase, AP.

also influences osteoblast proliferation and differentiation. However, alkaline phosphatase (AP) activity was found to be inhibited in osteoblast-like cells derived from fetal rat calvaria [1] but to be stimulated in an osteoblast-like rat osteosarcoma cell line [2]. Similar conflicting results were obtained with regard to cell proliferation [3,4]. These differences may be explained by the use of cultures in different stages of growth and maturation [5]. Furthermore, $1,25(OH)_2D_3$ treatment of primary cultures of osteoblast-like cells has been shown to produce an attenuation of the cAMP response to PTH [6].

We report here the effects of $1,25(OH)_2D_3$ on primary cultures of fetal rat osteoblast-like cells, which are known to possess receptors for $1,25(OH)_2D_3$ and PTH. These studies included potential effects on DNA content, AP activity and PTH-stimulated adenylate cyclase activity. To differentiate between effects on rapidly dividing cells and confluent cultures we performed the experiments in sparsely and densely seeded cultures.

Materials and Methods

Pregnant Wistar rats were obtained from TNO (Zeist, The Netherlands). MEM, fetal calf serum (FCS), L-glutamine, penicillin and streptomycin were purchased from Flow Laboratories. Collagenase, forskolin, synthetic bovine 1–34 PTH (bPTH 1–34) and bovine serum albumin (BSA) were obtained from Sigma. Vitamin D metabolites ($25(OH)D_3$, $1,25(OH)_2D_3$, $24,25(OH)_2D_3$) were donated by Hoffman La Roche (Mijdrecht, The Netherlands) and LEO Pharmaceuticals (Weesp, The Netherlands). Culture dishes (35 mm) were purchased from Greiner. The other reagents were of analytical grade.

Cell culture and treatment

Osteoblast-like cells (ROB-cells) were isolated from 20-day-old foetal rat calvaria according to the method of Boonekamp et al. [7]. After an initial treatment of the calvaria with isolation medium (137 mM NaCl, 2.7 mM KCl, 3 mM NaH_2PO_4, pH 7.2) supplemented with 4 mM EDTA for 2 × 10 min, the ROB cells were obtained by collagenase digestion (2 mg/ml isolation medium). The cells isolated after 2 × 20 min treatments with collagenase were harvested and washed with 15% FCS in isolation medium. Cells were plated in petri dishes (35 mm) at 5 and $20 \cdot 10^4$ cells in 1.5 ml of MEM with 10% FCS, 0.1 mg/ml glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37 °C in a humified 5% CO_2/95% air atmosphere. After 16–24 h, the medium was changed to 1 ml MEM with 2% charcoal-treated FCS, and aliquots of the vitamin D metabolites, dissolved in ethanol, were added to obtain the final concentrations indicated. The final ethanol concentration did not exceed 0.1% (v/v). The same amount of ethanol was added to the control cultures. Cells were maintained in culture for 3 days after the initiation of treatment with daily changes of medium and the vitamin D metabolite.

Cyclic AMP

Intracellular cAMP accumulation in triplicate cultures was stimulated by synthetic bPTH(1–34) in Hanks-Hepes buffer with 0.5% BSA. Maximal cAMP accumulation occurred at 2 min without, and at 5 min with, 0.2 mM IBMX. Subsequent experiments with PTH and forskolin were performed in the presence of 0.2 mM IBMX. After 5 min incubation cells were washed with 1 ml cold Hanks buffer and extracted with 1 ml 90% isopropanol. cAMP was measured in duplicate in 100 μl aliquots of the extracts by RIA [8].

DNA content and alkaline phosphatase activity

Cells were scraped off the dishes after being washed with PBS and were then sonicated in PBS with 0.1% Triton X-100. After centrifugation of the samples the supernatants were used for determination of AP activity and the pellets for estimation of DNA content. The assay of AP was done by the method of Lowry et al. [9] with *p*-nitrophenyl phosphate as a substrate. The amount of *p*-nitrophenol liberated was measured by its absorbance at 410 nm. The results were expressed in nmol *p*-nitrophenol formed per min per μg DNA (nmol PNP·min^{-1}·μg^{-1} DNA). The DNA content was determined fluorimetrically by the method of Johnson-Wint et al. [10] using calf thymus DNA as a standard.

Results

Effects of 1,25(OH)$_2$D$_3$ on DNA content and AP activity

A clear-cut stimulation of AP by 1,25(OH)$_2$D$_3$ was seen in sparse as well as dense cultures. Experiments were done with a broad range of 1,25(OH)$_2$D$_3$ concentrations (10^{-12}–10^{-8} M). The results shown in Fig. 1 only summarize the data obtained with the lowest effective concentration (10^{-10} M) and the highest concentration used (10^{-8} M). 10^{-8} M 1,25(OH)$_2$D$_3$ caused a considerable increase of AP activity. Only treatment of dense cultures that reached confluency on day 3 with a high (10^{-8} M) concentration 1,25(OH)$_2$D$_3$ resulted in a slight but reproducible decrease in

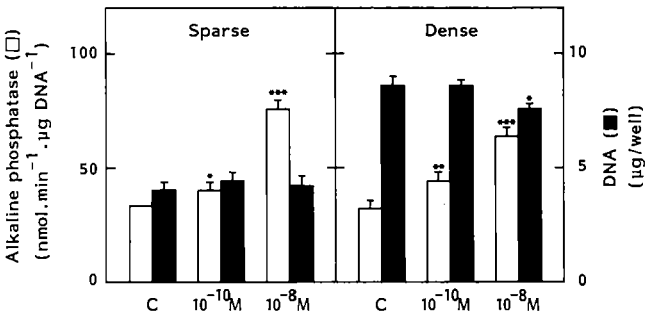

Fig. 1. Effect of 1,25(OH)$_2$D$_3$ on DNA content and AP activity in sparsely ($5 \cdot 10^3$ cells/cm^2) and densely ($20 \cdot 10^3$ cells/cm^2) seeded cultures. Values are means ± SD of four experiments. Significance versus control: (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

Table 1

Comparison of the effects of different vitamin D derivates on growth and alkaline phosphatase

Treatment	DNA (μg/well)	AP (nmol PNP·min^{-1}·μg^{-1} DNA)
Control	8.5 ± 0.4	33.0 ± 2.1
1,25(OH)$_2$D$_3$ (10^{-8} M)	7.4 ± 0.3*	76.2 ± 2.4**
24,25(OH)$_2$D$_3$ (10^{-8} M)	8.4 ± 0.3	36.0 ± 4.2
25(OH)D$_3$ (10^{-8} M)	8.6 ± 0.3	40.0 ± 4.1

Densely seeded ROB-cells ($20 \cdot 10^3$/cm^2) were cultured for 3 days in the presence of different vitamin D metabolites. Values are means ± SEM of five cultures each. Significance versus control: (*) $P < 0.05$; (**) $P < 0.001$.

DNA content. Parallel experiments with 25-(OH)D$_3$ or 24,25(OH)$_2$D$_3$ did not show an increase of AP at 10^{-8} M (Table 1).

Effect of 1,25(OH)$_2$D$_3$ on PTH and forskolin stimulated cAMP response

Treatment of ROB cells for 3 days with 1,25(OH)$_2$D$_3$ produced a marked attenuation of the intracellular cAMP response to PTH (Fig. 2). The lowest effective 1,25(OH)$_2$D$_3$ concentration was the same as for the effect on AP, i.e., 10^{-10} M. The basal level of cAMP was not consistently different in control and 1,25(OH)$_2$D$_3$ treated cultures. There was no change in the EC_{50} of PTH. If the results are expressed as pmol cAMP per μg DNA, up to 10^{-8} M 1,25(OH)$_2$D$_3$ did not alter the EC_{50} of PTH, not even in the dense cultures. When ROB cells were cultured with 1,25(OH)$_2$D$_3$ (10^{-8} M) and the cAMP response to PTH was measured at several time points thereafter, the decrease of the responsiveness became apparent between 8 and 24 h (Fig. 3). In Fig. 4 it is shown that, at both cell densities used, 1,25(OH)$_2$D$_3$ caused a decline in the PTH-stimulated cAMP response. However, at low density, where the cultures were not confluent at the time of PTH stimulation, the cAMP response was higher both in control and 1,25(OH)$_2$D$_3$ treated cultures.

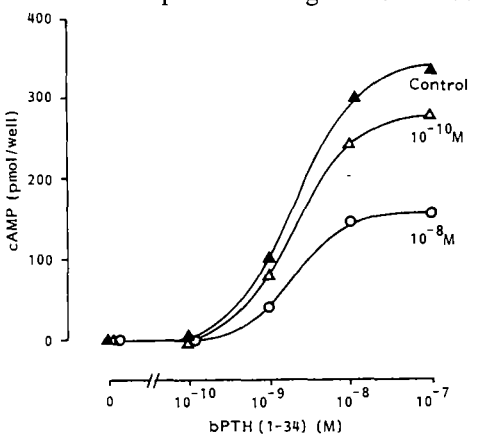

Fig. 2. Effect of 1,25(OH)$_2$D$_3$ on cAMP response to PTH in densely ($20 \cdot 10^3$ cells/cm^2) seeded cultures. Values are means of three experiments. (All SEM fall within the size of the symbols.)

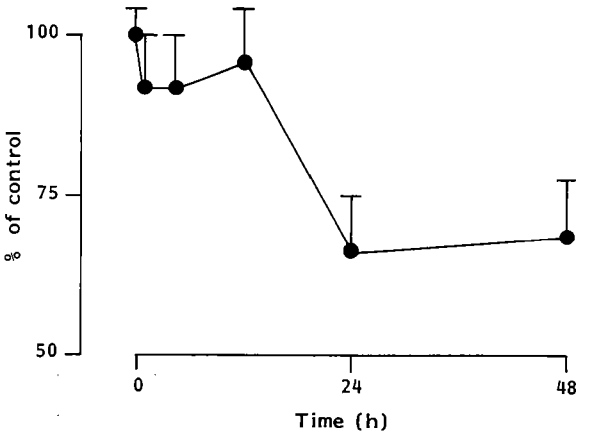

Fig. 3. Time course of the $1,25(OH)_2D_3$ effect on the PTH ($5 \cdot 10^{-9}$ M) stimulated cAMP response in densely ($20 \cdot 10^3$ cells/cm^2) seeded cultures. Values are means ± SEM of five cultures.

Since it has been suggested that the diterpene forskolin can stimulate the catalytic unit of the adenylate cyclase system directly [11,12], we examined its effect on cAMP accumulation and the possible interaction of $1,25(OH)_2D_3$ with this action of forskolin. As shown in Fig. 5 the response of cAMP to forskolin alone was not influenced by treatment with $1,25(OH)_2D_3$ (10^{-8} M) for 3 days. Figure 6 demonstrates that forskolin raised the PTH ($5 \cdot 10^{-9}$ M) stimulated cAMP production substantially even at concentrations of forskolin ($<10^{-6}$ M) that by themselves did not produce a rise in cAMP. Although increasing concentrations of forskolin were not able to correct the $1,25(OH)_2D_3$ induced attenuation of the PTH-stimulated cAMP response completely, a significant decrease in the ratio of cAMP values in control over $1,25(OH)_2D_3$ treated cultures was observed (Fig. 6, inset).

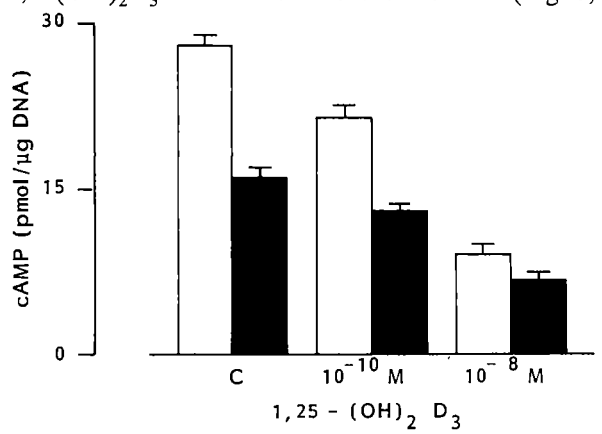

Fig. 4. Effect of $1,25(OH)_2D_3$ on PTH ($5 \cdot 10^{-9}$ M) stimulated cAMP response in sparsely and densely seeded cultures. Seeding density: (□) $5 \cdot 10^3$ cells/cm^2; (■) $20 \cdot 10^3$ cells/cm^2. Values are means ± SEM of three to four experiments.

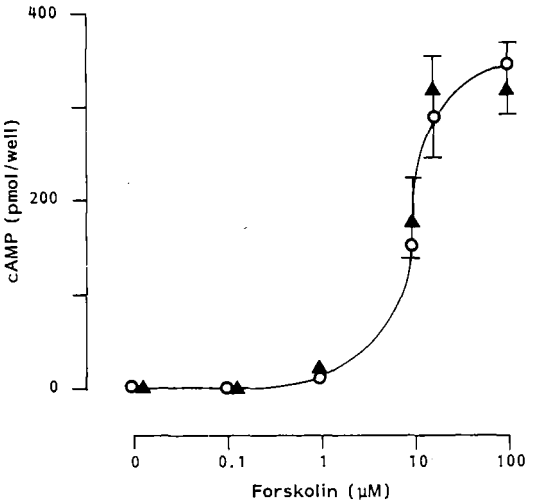

Fig. 5. Effect of $1,25(OH)_2D_3$ on forskolin (0–100 μM) stimulated cAMP response in densely ($20 \cdot 10^3$ cells/cm^2) seeded cultures. (○) control; (▲) 10^{-8} M $1,25(OH)_2D_3$. Values are means ± SEM of three experiments. No significant difference between control and $1,25(OH)_2D_3$ treated cultures.

Discussion

In previous studies effects of $1,25(OH)_2D_3$ on growth, AP and modulation of the cAMP response to PTH have been reported separately and mostly in osteosarcoma cell lines. In this study the mechanism of action of $1,25(OH)_2D_3$ on these parameters have been studied together in primary cultures of ROB-cells. The findings

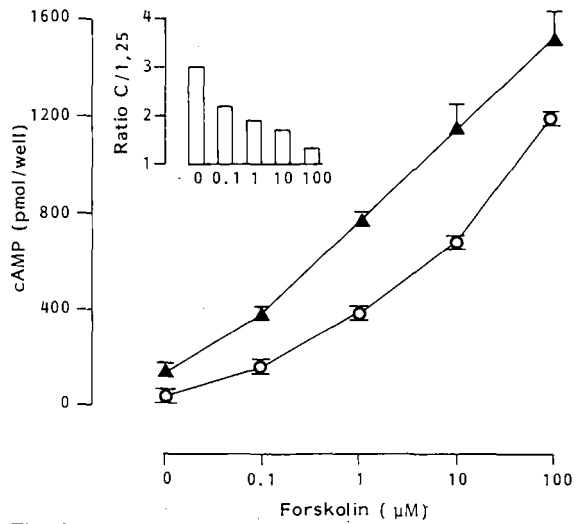

Fig. 6. cAMP response to forskolin (0–100 μM) added together with a fixed concentration of PTH ($5 \cdot 10^{-9}$ M) in control (▲) and $1,25(OH)_2D_3$ 10^{-8} treated cultures (○). Values are means ± SEM of two to three experiments. Inset. Ratio control over $1,25(OH)_2D_3$ plotted against forskolin concentration. The data on cAMP were first corrected for DNA content before this ratio was calculated.

presented show that, irrespective of cell density, $1,25(OH)_2D_3$ treatment of ROB cultures results in a pronounced increase of AP activity and causes an attenuation of the cAMP response to PTH. Under the conditions used the growth inhibiting effect of $1,25(OH)_2D_3$ was only found in dense cultures at a high $1,25(OH)_2D_3$ concentration (10^{-8} M). This finding differs to some extent from the results reported by Chen et al. [4], who found a decrease in both sparse and dense ROB cultures (from Sprague-Dawley rats), although more pronounced in dense cultures. This discrepancy is probably not due to lower levels of exogenous growth factors in our case, as Chen et al. state that in serum-free cultures the growth inhibition by $1,25(OH)_2D_3$ is even more pronounced. A methodological difference that might be important concerns the daily change of culture medium in our experiments. Anyway, it appears that for the growth inhibition by $1,25(OH)_2D_3$ cell–cell interactions are essential.

The effect of $1,25(OH)_2D_3$ on AP, used as a marker of differentiation of osteoblasts, appears to be specific, since other vitamin D metabolites produce a slight increase of AP only at 10^{-8} M. This is in agreement with observations that in several osteosarcoma cell lines the $1,25(OH)_2D_3$ induced stimulation of AP is mediated via its specific receptor and involves genome activation and de novo protein synthesis [2,13]. The same experiments carried out with a late passage of the rat osteosarcoma cell line UMR 106 showed a high basal AP level which could not be stimulated by $1,25(OH)_2D_3$ up to 10^{-8} M [14]. In this respect our results with primary cultures of ROB-cells are comparable to the results obtained by Spiess et al. [15] in those cells cloned from ROS 17/2.8 osteosarcoma cells, which exhibited low basal AP activity, while our results with UMR-106 cells are more in line with the high AP clones reported by the same authors.

The $1,25(OH)_2D_3$ induced 'desensitization' of the adenylate cyclase complex to PTH has been reported previously in both primary cultures of fetal osteoblast-like cells [6] and in osteosarcoma cell lines of the osteoblastic phenotype [16,17]. It is unlikely that $1,25(OH)_2D_3$ exerts its effects on the cyclic nucleotide phosphodiesterase because the experiments were performed in the presence of the phosphodiesterase inhibitor IBMX. Furthermore, the dose-response curves showed no significant change of the apparent EC_{50} of PTH, so that a decrease of the affinity of PTH for its receptor is also improbable. At this stage it is impossible to definitely demonstrate or exclude an effect of $1,25(OH)_2D_3$ on the number of PTH receptors.

Under the conditions described $1,25(OH)_2D_3$ does not inhibit the cAMP accumulation in reaction to forskolin alone. Increasing concentrations of this agent in combination with PTH show not only the known synergistic effect [12] on cAMP accumulation, but also the ability to overcome in part the $1,25(OH)_2D_3$ induced attenuation of the cAMP response to PTH. Although the direct stimulation of the adenylate cyclase by forskolin does not seem to require the guanine nucleotide binding

protein (N_s) [11], there is also evidence for a role of the N_s in the synergistic activity of forskolin in the presence of hormone [18,19]. This is illustrated by the potentiation of the effect of PTH by concentrations of forskolin that by themselves do not stimulate cAMP production (Fig. 6). Since the inhibitory effect of $1,25(OH)_2D_3$ treatment on PTH-stimulated cAMP production is alleviated by forskolin we speculate that $1,25(OH)_2D_3$ interferes with the N_s or the interaction of the N_s with the catalytic unit and that this interaction is restored in the presence of forskolin.

Other factors that have to be considered are $1,25(OH)_2D_3$ induced changes in intracellular Mg or Ca. Mg is known to be necessary in the activation of the adenylate cyclase [17]. On the other hand, $1,25(OH)_2D_3$ might inhibit adenylate cyclase activity by reducing intracellular exchangeable Ca [20,21].

Finally, it is important to emphasize that the response of cAMP to PTH as well as its sensitivity to $1,25(OH)_2D_3$ are inversely related to cell density (Fig. 4). Therefore, irrespective of the presence of $1,25(OH)_2D_3$, factors related to growth appear to be able to modulate the cAMP response. This in itself might represent an aspect of differentiation.

Acknowledgement

This study was supported by a grant from the Dutch Kidney Foundation (no. C83.407).

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APPENDIX PAPER 2

THE INFLUENCE OF ALBUMIN ON VITAMIN D METABOLISM IN FETAL CHICK OSTEOBLAST-LIKE CELLS

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Received October 17, 1984

SUMMARY Incubation of osteoblast-like cells with [3H]25-(OH)D3 and varying bovine serum albumin (BSA) concentrations resulted in a dramatic change in the accumulation of 1,25-(OH)2D3 and 24,25-(OH)2D3 in the medium. At 0.1% BSA 1,25-(OH)2D3 formation was transient and 24,25-(OH)2D3 was the main product after 3 h. At 2% BSA accumulation of 1,25-(OH)2D3 was sustained whereas 24,25-(OH)2D3 formation was suppressed. At low BSA levels added [3H]1,25-(OH)2D3 was rapidly metabolized to 1,24,25-(OH)3D3 and more polar metabolites. The effect of increasing BSA concentrations on 25-(OH)D3 metabolism was mimicked by addition of cycloheximide. This indicates that high BSA levels prevent the induction of 24-hydroxylase activity in this system, probably by lowering of the free 25-(OH)D3 concentration. The accumulation of 1,25-(OH)2D3 from 25-(OH)D3 not only depends on the 1α-hydroxylase activity, but also on the further metabolism of 1,25-(OH)2D3 by 24-hydroxylation.

The synthesis of $1,25\text{-}(OH)_2D_3$ from $25\text{-}(OH)D_3$ has long been considered to be confined to the kidney (1). More recent studies, however, showed evidence that cells from a number of other tissues also have the ability to convert $25\text{-}(OH)D_3$ to $1,25\text{-}(OH)_2D_3$ and $24,25\text{-}(OH)_2D_3$. This has been observed in fetal chicken calvarial cells (2,3), chicken and rabbit cartilage (4), human bone cells and human osteogenic sarcoma (5), chick chorioallantoic membrane (6) and rat placenta (7). That extrarenal $1,25\text{-}(OH)_2D_3$ production occurs in humans is supported by the observation of low but significant levels of $1,25\text{-}(OH)_2D_3$ in anephric patients (8).

Since knowledge about the production of vitamin D_3 metabolites by bone cells and its regulation may be crucial for understanding the effect of vitamin D on the skeleton, we studied the conversion of vitamin D metabolites in fetal chicken osteoblast-like cells (OB-cells). In this paper we report that primary cultures of OB-cells are capable to convert $25\text{-}(OH)D_3$ to more polar metabolites,

Abbreviations employed are: 25-hydroxy-vitamin D3 (25-(OH)D3); 1,25-dihydroxy-vitamin D3 (1,25-(OH)2D3; 24,25-dihydroxy-vitamin D3 (24,25-(OH)2D3); 1,24,25-trihydroxy-vitamin D3 (1,24,25-(OH)3D3).

including $1,25\text{-(OH)}_2D_3$ and $24,25\text{-(OH)}_2D_3$, and that addition of increasing concentrations of bovine serum albumin (BSA) or of cycloheximide profoundly change $25\text{-(OH)}D_3$ metabolism.

MATERIALS AND METHODS [26,27-3H] 25-(OH)D3 (19.2 Ci/mmol), [23,24-3H] 1,25-(OH)2D3 (85 Ci/mmol) and [23,24-3H]24,25-(OH)2D3 (64 Ci/mmol) were obtained from Amersham. Standards of 25-(OH)D3, 1,25-(OH)2D3 and 24,25-(OH)2D3 were donated by Hoffmann-La Roche (Mijdrecht, The Netherlands) and Duphar (Weesp, The Netherlands). 1,24,25-(OH)3D3 was a generous gift of Dr. R. Bouillon (Catholic University, Louvain, Belgium), The following materials were from Sigma: BSA (fraction V), collagenase type I, α-tosyl-lysyl-chloromethane and HEPES. Eagle's MEM, embryonic extract (EE), penicilline and streptomycine sulphate were obtained from Flow Laboratories. Fetal calf serum (FCS) and the culture dishes (35 mm) were purchased from Greiner. The other reagents were of analytical grade.

Cell culture and incubation procedures. Osteoblast-like cells (OB-cells) were isolated from 18 day-old chick embryo calvaria with collagenase (2 mg/ml isolation medium (9)) as previously described (10). The isolated cells were washed with 15% (v/v) FCS in Hanks-HEPES solution, centrifuged, and then suspended in 10 ml cultivation fluid consisting of Eagle's MEM (buffered with Earle's BSS, pH 7.4), 10% FCS, 5% EE, 0.1 mg/ml glutamine, 0.05 mg/ml ascorbic acid, 100 units/ml penicilline and 100 μg/ml streptomycine. The cells were then seeded in petri dishes and cultured in a moist atmosphere of 5% CO2 in air (37 C) until the cultures reached confluency (5-6 days). Media were changed every two days with 1 ml of the above-mentioned medium, without EE. At least 16 h prior to incubation with substrate the growth medium was replaced by Eagle's MEM (Ca++ 1,25 mM) containing no FCS, but BSA in concentrations ranging from 0.1 to 2%. The reaction was started by the addition of the radioactive vitamin D metabolites dissolved in ethanol (final concentration <0.1%) as indicated in the legends of the figures. The incubations (0.5-3 h) were carried out with the culture dishes placed on a slightly angled, slowly rotating plate, which enhanced the rate of metabolism considerably. At the end of the incubations the medium was removed and extracted with diethyl ether. The lipid extract was subjected to high pressure liquid chromatography (HPLC) on a 0.46 x 25 cm CPtmSpher Silica column (Chrompack) using hexane-isopropanol (91 : 9) as the solvent at a flow rate of 1.8 ml/min or on a 0.46 x 25 cm Zorbax CN column (Du Pont) using hexane-isopropanol-methanol (94 : 5: 1) as the solvent at a flow rate of 1.3 ml/min. The radioactive vitamin D metabolites were identified by their retention time after calibration with standard 25-(OH)D3, 24,25-(OH)2D3, 1,25-(OH)2D3 and 1,24,25-(OH)3D3. Aqueous periodate treatment of putative 1,25-(OH)2D3 and 24,25-(OH)2D3 was performed according to Tanaka et al. (11). At 0.1-2% BSA the radioactivity recovered from the incubation medium varied from 60-90% for [3H]25-(OH)D3 and from 75-90% for [3H]1,25-(OH)2D3, respectively. The DNA content of the cultures was determined according to the method of Johnson-Wint et al. (12). Because of the narrow range of the DNA content per dish among different cultures (15 ± 2.3 μg) the results were expressed as pmol per sample or, where indicated, as the percentage radioactivity recovered.

Binding of vitamin D3 metabolites at different BSA concentrations. Tritiated 25-(OH)D3, 24,25-(OH)2D3 or 1,25-(OH)2D3 (0.5-10 nM) were incubated for 1 h at 37C with different BSA concentrations (0.1-4%) in serum-free medium. At the end of the incubations protein-bound and free vitamin D were separated by charcoal-dextran adsorption (13), and the bound fraction was counted for radioactivity.

RESULTS

Identification of $1,25\text{-(OH)}_2D_3$ and $24,25\text{-(OH)}_2D_3$ formed by the OB-cells. We first examined the ability of primary cultures of OB-cells to metabolize

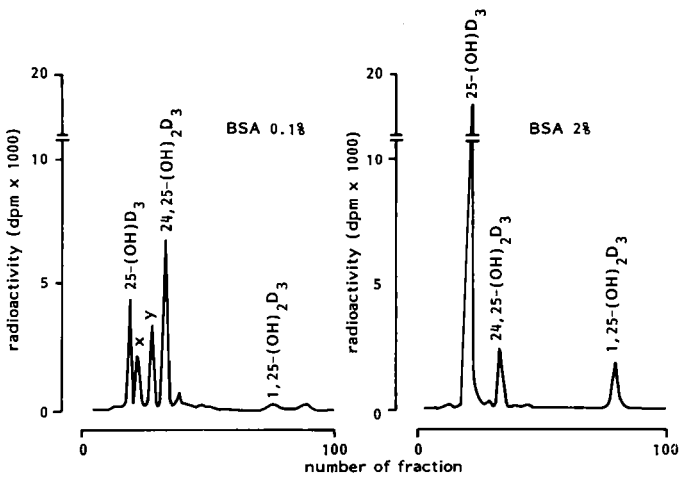

Fig.1 Representative metabolite profiles at 0.1% and 2% BSA after 3 h incubation of OB-cells with 10 nM [3H]25-(OH)D_3. (CPtmSpher Silica column; hexane-isopropanol = 91 : 9; fraction volume 0.45 ml).

25-(OH)D_3 in a 0.1 and 2% BSA medium. After 3 h incubation and subsequent processing of the medium we obtained the typical elution profiles shown in Fig.1. No metabolites other than 25-(OH)D_3 were found if the incubations with [^{3}H]25-(OH)D_3 were performed without cells or with cells killed with glutaraldehyde. Of the polar metabolites formed two were identified as 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ on the basis of their comigration with authentic standards on HPLC in two different systems (Silica and Zorbax CN column). After periodate treatment 90-95% of the radioactivity in the 24,25-$(OH)_2D_3$ and 2-5% of that in the 1,25-$(OH)_2D_3$ region was lost.

25-(OH)D_3 metabolism at different medium BSA concentrations. Incubation of the OB-cells for 3 h with 25-(OH)D_3 at 0.1% BSA resulted in the accumulation of a large amount of 24,25-$(OH)_2D_3$ and a small amount of 1,25-$(OH)_2D_3$, while at 2% BSA a more pronounced 1,25-$(OH)_2D_3$ accumulation was accompanied by low levels of 24,25-$(OH)_2D_3$ (Fig.2). The accumulation of two other metabolites, denoted X and Y in Fig.1, is also lowered by raising the medium BSA concentration. Time course experiments demonstrated the transient nature of the accumulation of 1,25-$(OH)_2D_3$ in the presence of 0.1% BSA, while at 2% BSA an initially slower, but more sustained rise of this derivative was found (Fig.3). The decline of 1,25-$(OH)_2D_3$ after 2 h in the presence of 0.1% BSA was accompanied by an acceleration of the 24,25-$(OH)_2D_3$ accumulation. At 2% BSA the concentration of 24,25-$(OH)_2D_3$ in the medium remained low.

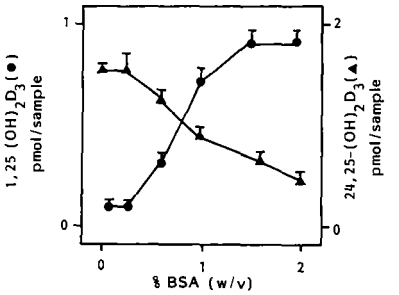

Fig.2 Accumulation of 1,25-(OH)2D3 (●) and 24,25-(OH)2D3 (▲) at 0.1-2% BSA after 3 h incubation with 10 nM [3H]25-(OH)D3. Values represent the means ± SEM of 3 different experiments.

1,25-$(OH)_2D_3$ metabolism at different medium BSA concentrations. Further 24-hydroxylation of the product may be the underlying mechanism for the transient accumulation of 1,25-$(OH)_2D_3$ at 0.1% BSA. To simulate the above-mentioned experiments, cells were incubated for 3 h with a low concentration of labeled 1,25-$(OH)_2D_3$ (0.5 nM) together with 10 nM unlabeled 25-$(OH)D_3$. Recently, we have shown that in OB-cells, 1,25-$(OH)_2D_3$ is rapidly converted to 1,24,25-$(OH)_3D_3$ and other more polar metabolites (14). In Fig.4 the influence of varying medium BSA concentrations on the accumulation of 1,24,25-$(OH)_3D_3$ is seen. At 0.1% BSA considerable conversion to 1,24,25-$(OH)_3D_3$ was found, which was progressively inhibited by increasing BSA concentrations. Non-ether-extractable radioactivity was affected in the same manner as the 1,24,25-$(OH)_3D_3$ accumulation.

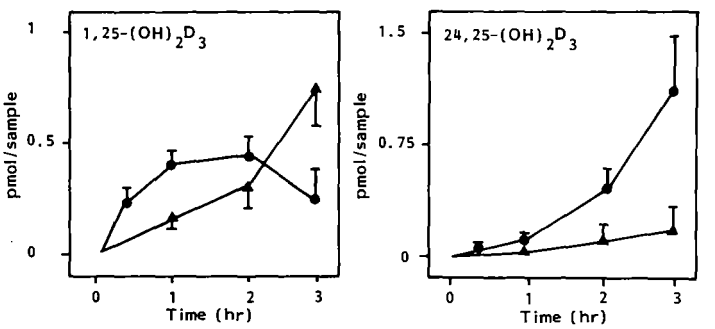

Fig.3 Effect of incubation time on the metabolism of [3H]25-(OH)D3 (10 nM) at 0.1% (●) and 2% (w/v) BSA (▲) after incubation for 0.5-3 h. Values represent the means ± SEM of 3 different experiments.

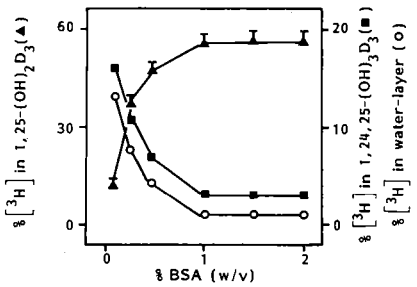

Fig.4 1,25-(OH)2D3 metabolism at 0.1-2% (w/v) BSA after 3 h incubation with 0.5 nM [3H]1,25-(OH)2D3 and 10 nM 25-(OH)D3. 1,25-(OH)2D3 (▲) and 1,24,25-(OH)3D3 (■) were separated by HPLC. The water-layer (○) represents the non-diethyl ether-extractable fraction. Values represent the means ± SEM of at least 2 experiments.

Effect of cycloheximide. To assess whether the 24-hydroxylase activity observed in our experiments requires de novo protein synthesis, we studied the metabolism of 25-(OH)D_3 in the presence and absence of cycloheximide. The experiments were performed at 0.1% BSA because of the pronounced 24,25-$(OH)_2D_3$ accumulation under these circumstances. Incubation of OB-cells with cycloheximide (1 µg/ml) for 3 h resulted in significantly lower levels of 24,25-$(OH)_2D_3$ and significantly higher levels of 1,25-$(OH)_2D_3$ (Table I). Under these circumstances a decline of the peaks X and Y (cf Fig.1) was found also (not shown).

Binding of vitamin D_3 metabolites at different BSA concentrations. The effect of BSA may be secondary to changes in the availability of 25-(OH)D_3 and its metabolites. Figure 5 shows the progressive increase in the binding of the

Table I

Effect of cycloheximide on 25-(OH)D_3 metabolism in OB-cells

	Accumulation of [^{3}H]25-(OH)D_3 metabolites	
Addition	1,25-$(OH)_2D_3$ pmol/sample	24,25-$(OH)_2D_3$ pmol/sample
none	0.13 ± 0.02	1.09 ± 0.04
cycloheximide	0.33 ± 0.09*	0.37 ± 0.02*

OB-cells were incubated for 3 h at 0.1% BSA with 10 nM [^{3}H]25-(OH)D_3 in the presence or absence of 1 µg/ml cycloheximide. The media of 5 parallel incubations per experiments were analysed as described under Materials and Methods. Values represent the means ± SEM of 2 experiments. (* $p < 0.001$ vs control)

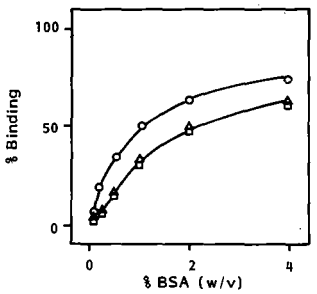

Fig.5 Binding of 25-(OH)D3, 24,25-(OH)2D3 and 1,25-(OH)2D3 to BSA. Serum-free medium with 0.1-4% (w/v) BSA was incubated for 1 h with 0.5 nM [3H]25-(OH)D3 (○), [3H]1,25-(OH)2D3 (□) or [3H]24,25-(OH)2D3 (△). Values represent the means ± SEM of at least 2 experiments.

different vitamin D derivatives to increasing BSA concentrations. Highest binding is observed with 25-(OH)D_3, while 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ have equal affinity for BSA. The binding of all three metabolites was not significantly influenced by increasing their concentration from 0.5 to 10 nM.

DISCUSSION

The observation that fetal chicken calvarial cells are able to metabolize 25-(OH)D_3 to 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ has been interpreted as a possible means of local regulation of the response to vitamin D (2). However, it was not clear to what extent the use of a protein-free medium might have influenced the ability of these cells to metabolize 25-(OH)D_3 via the different pathways. We performed our experiments in the presence of a range of BSA concentrations. We found that fetal chicken osteoblast-like cells metabolize 25-(OH)D_3 both at 0.1% and 2% BSA (Fig.1), although marked differences in the accumulation of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ are seen (Fig.2). The striking differences in the time-course experiments at 0.1% and 2% BSA (Fig.3) suggest that factors such as the availability of the substrate to the enzymes and the rates of further conversion of the products alter the accumulation of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$. The high free substrate concentration in the presence of 0.1% BSA (Fig.5) initially appears to result in a high rate of synthesis of the two dihydroxy-metabolites (Fig.3). However, once a significant production of 24,25-$(OH)_2D_3$ takes place the level of 1,25-$(OH)_2D_3$ falls probably due to further hydroxylation in the 24 position. This is supported by the observation that a considerable proportion of added 1,25-$(OH)_2D_3$ is converted to 1,24,25-$(OH)_3D_3$. The latter conversion is decreased at higher BSA concentrations (Fig.4), which is in agreement with the ultimately higher accumulation of 1,25-$(OH)_2D_3$ from 25-(OH)D_3 in the presence of 2% BSA.

It is clear from these results that 24-hydroxylation represents a major step in the metabolism of 25-(OH)D_3 and 1,25-$(OH)_2D_3$, which however is fully expressed only at low ambient albumin concentrations, i.e. high free substrate concentrations. It is also evident that once 24,25-$(OH)_2D_3$ is allowed to accumulate other metabolites (cf. the peaks X and Y in Fig.1) appear also. These compounds probably are the 24-oxo- and 23-hydroxy-derivatives of 25-$(OH)_2D_3$, recently identified in perfusion studies of rat kidney (15). For 1,25-$(OH)_2D_3$ we also propose further conversion by the C_{23}- and C_{24}-oxidation pathways, as the loss of tritium from 23,24-tritiated 1,25-$(OH)_2D_3$ into the medium appeared to be much higher than one would expect from 24-hydroxylation alone (Fig.4). This hypothesis is supported by the recent observation of such a pathway in intestinal 1,25-$(OH)_2D_3$ metabolism (16).

Other workers (2) using comparable substrate concentrations have concluded that the rates of production of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ in fetal chicken calvarial cells are linear for up to 4 h. We observed that the induction of the 24-hydroxylase, which is dependent upon de novo protein synthesis (Table I), is one of the factors that cause the non-linearity in the accumulation of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ in our experiments. The fact that incubation with cycloheximide also results in a higher level of 1,25-$(OH)_2D_3$ formed from 25-(OH)D_3 again indicates the formation of 1,24,25-$(OH)_3D_3$ at low BSA concentrations in the standard incubations. Because 1,25-$(OH)_2D_3$ is known as a potent inductor of 24-hydroxylase activity (2,17,18) it is attractive to speculate that the (preceeding) accumulation of this compound is responsible for the increase in 24-hydroxylase activity. Induction of 24-hydroxylase activity by 25-(OH)D_3 itself is, however, not excluded (19).

We do not know whether or not the 1α- and 24-hydroxylases are located in the same type of cell in our culture. It is important to note that although primary cultures of OB-cells retain several characteristics of osteoblasts (10), a significant admixture of other cells, especially fibroblasts, may occur. Although fibroblasts do not contain basal 1α- and 24-hydroxylase activities (2), a rapid induction of 24-hydroxylase activity by 1,25-$(OH)_2D_3$ in these cells has been reported (20,21). A similar induction could also play a role in our experiments. Therefore, the significance of 24-hydroxylation as a major mechanism of inactivation of 1,25-$(OH)_2D_3$ in bone tissue remains to be clarified. The main question raised by our study is to what extent the metabolism of 25-(OH)D_3 in OB-cell cultures reflect the in vivo situation. Our results strongly suggest that interpretation of such studies is not possible without knowledge of the effects of vitamin D-binding proteins.

ACKNOWLEDGEMENT: This study was supported by a grant from the Dutch Kidney Foundation (no. C 83.407).

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APPENDIX PAPER 3

Effect of ketoconazole on metabolism and binding of 1,25-dihydroxyvitamin D-3 by intact rat osteogenic sarcoma cells

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(Received 13 May 1987)

Key words: 1,25-Dihydroxyvitamin D-3 metabolism; 1,25-Dihydroxyvitamin D-3 receptor; Receptor up-regulation; Ketoconazole; Vitamin D-3; (Rat osteogenic sarcoma cell)

The antifungal imidazole, ketoconazole, was tested for effects on 1,25-dihydroxyvitamin D-3 ($1,25\text{-}(OH)_2D_3$) metabolism and binding in intact osteoblast-like osteogenic sarcoma cells (UMR-106). Ketoconazole inhibited the C-24 oxidation of $1,25\text{-}(OH)_2D_3$ in a dose-dependent manner. Furthermore, inhibition of $1,25\text{-}(OH)_2D_3$ metabolism by ketoconazole resulted, after a lag time of 2 h, in a sharp increase of receptor-bound $1,25\text{-}(OH)_2D_3$. The data suggest that the self-induced $1,25\text{-}(OH)_2D_3$ metabolism may play an important role in controlling the intracellular levels of and, consequently, receptor occupancy by the active form of vitamin D. Furthermore the results are compatible with the existence of a homologous up-regulation of the $1,25\text{-}(OH)_2D_3$-receptor.

Introduction

Ketoconazole is a broad spectrum, antifungal drug and has become a major therapeutic modality in the treatment of mycotic infections. However, it has recently been observed to inhibit both adrenal and testicular steroidogenesis [1–3]. In addition, there are also reports showing that ketoconazole inhibits the 24-hydroxylation of 25-hydroxyvitamin D-3 [3,4]. Recent evidence suggests that imidazoles, like ketoconazole, inhibit cytochrome *P*-450 mixed function oxidases, probably by interacting with the heme iron of the cytochrome [5,6].

We have developed a system to measure simultaneously 1,25-dihydroxyvitamin D-3 ($1,25\text{-}(OH)_2D_3$) receptor binding and metabolism in intact osteoblast-like sarcoma cells (UMR-106). Recently, we found that the time course of specific binding of $1,25\text{-}(OH)_2D_3$ measured in UMR-106 cells is characterized by (a) an ascending phase, representing association with receptor, (b) a maximum at 90–120 min and (c) a rapid descending phase closely associated with a decrease of medium $1,25\text{-}(OH)_2D_3$ due to metabolism of the hormone [7]. In this study, we provide experimental evidence that inhibition of the self-induced metabolism of $1,25\text{-}(OH)_2D_3$ by ketoconazole not only prevents the descending phase, but also results in a time-dependent increase of specific binding of $1,25\text{-}(OH)_2D_3$.

Abbreviations: $1,25\text{-}(OH)_2D_3$, 1,25-dihydroxyvitamin D-3; $1,24,25\text{-}(OH)_3D_3$, 1,24,25-trihydroxyvitamin D-3; $24\text{-oxo-}1,25\text{-}(OH)_2D_3$, 24-oxo-1,25-dihydroxyvitamin D-3; $24\text{-oxo-}1,23,25\text{-}(OH)_3D_3$, 24-oxo-1,23,25-trihydroxyvitamin D-3.

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Materials and Methods

[23,24-^{3}H]1,25-$(OH)_2D_3$ (102 Ci/mmol) or [26,27-^{3}H]1,25-$(OH)_2D_3$ (100 Ci/mmol) was obtained from Amersham International. Ketoconazole was purchased from Janssen Pharmaceuticals (Belgium). Synthetic 1,25-$(OH)_2D_3$ was donated by Hoffman-La Roche (Mijdrecht, The Netherlands). 1,24,25-$(OH)_3D_3$ was a generous gift of Dr. R. Bouillon (Catholic University, Louvain, Belgium). Bovine serum albumin (fraction V) was from Sigma, while minimum essential medium, glutamine, penicilline and streptomycine were obtained from Flow Laboratories. Fetal calf serum and the culture dishes (35-mm petri dishes) were purchased from Greiner. The other reagents were of analytical grade. UMR-106 cells were supplied by Dr. M.P.M. Hermann-Erlee (Laboratory of Cell Biology, University of Leiden, Leiden, The Netherlands).

Cell culture. Rat osteogenic sarcoma cells (UMR-106) were grown in minimum essential medium supplemented with 2 mM glutamine, 100 μg/ml streptomycine and 5% fetal calf serum.

Incubation procedure. After a 16-h period of preincubation in minimum-essential medium with 2% charcoal-treated fetal calf serum [8], the confluent cultures (approx. 10^5 cells/cm^2) were incubated in serum-free minimum essential medium with 0.1% bovine serum albumin, varying concentrations of ketoconazole (10^{-8}–10^{-4} M) and the desired amount of [23,24-^{3}H]1,25-$(OH)_2D_3$ or [26,27-^{3}H]1,25-$(OH)_2D_3$ with or without a 200-fold excess of unlabeled 1,25-$(OH)_2D_3$. The incubations were carried out for 0–4 h at 37 °C with the cultures placed on a slightly angled, slowly rotating plate. At the end of each incubation period the medium was removed. The cells were additionally incubated for 10 min at room temperature with Hanks' balanced salt solution containing 2% bovine serum albumin and then rinsed twice more with ice-cold Hanks' balanced salt solution alone. The medium and the Hanks' balanced salt solutions were pooled and extracted with diethyl ether, resulting in two separate layers; an ether-soluble lipid layer and a non-ether-extractable water layer. The water layer was frozen, after which the ether layer was decanted. The lipid extract was subjected to HPLC on a CPtm Spher Silica column (10 × 0.3 cm, Chrompack) with hexane/isopropanol/methanol (88 : 10 : 2) as solvent at a flow rate of 0.6 ml/min. Radioactivity eluting with 1,25-$(OH)_2D_3$ and 1,24,25-$(OH)_3D_3$ was quantitated. The radioactivity in the non-ether-extractable water fraction, which represents the loss of the tritium label through C-24 oxidation of 23,24-tritiated 1,25-$(OH)_2D_3$ [9], was also quantitated. For the measurement of cellular accumulation of 1,25-$(OH)_2D_3$, cells were solubilized in 0.1 M NaOH, neutralized with acetic acid and counted for radioactivity. Specific cellular accumulation was estimated as the difference between cellular accumulation of [^{3}H]1,25-$(OH)_2D_3$ alone and non-specific (with excess unlabeled 1,25-$(OH)_2D_3$) cellular accumulation.

Receptor-bound [^{3}H]1,25-$(OH)_2D_3$ was extracted on ice in 200 μl of 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 [10]. The extract was adjusted to 60 mM KCl and 0.02% Triton X-100 by 5-fold dilution with buffer lacking these constituents. Receptor-bound 1,25-$(OH)_2D_3$ was separated from unbound sterol using charcoal adsorption [11].

Results and Discussion

As shown before, monolayers of UMR-106 cells are very active in metabolizing 1,25-$(OH)_2D_3$. This metabolism is induced by the presence of the hormone and can be blocked by inhibition of mRNA or protein synthesis [7]. To investigate whether ketoconazole also has the ability to inhibit the self-induced metabolism of 1,25-$(OH)_2D_3$, cells were incubated for 4 h with 0.5 nM [^{3}H]1,25-$(OH)_2D_3$ and varying concentrations ketoconazole. As depicted in Fig. 1a, ketoconazole caused a dose-dependent inhibition of 1,25-$(OH)_2D_3$ metabolism, resulting in an increase of medium 1,25-$(OH)_2D_3$ concentration and a decrease of the non-ether-extractable radioactivity in the water fraction. At the highest concentration used (10^{-4} M), ketoconazole elicits a decrease of approx. 70% of 1,25-$(OH)_2D_3$ metabolism as calculated from the decrease of radioactivity in the water fraction.

As shown in Fig. 1b, inclusion of increasing

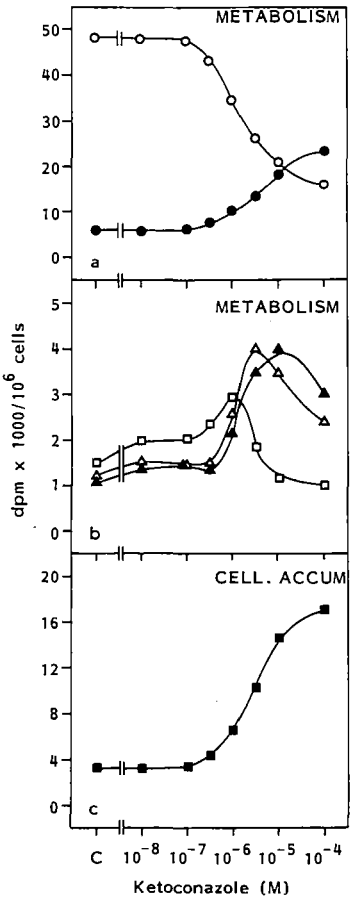

Fig. 1. Effect of ketoconazole on metabolism and cellular accumulation of [23,24-^{3}H]1,25-$(OH)_2D_3$. Cells were incubated for 4 h with 0.5 nM [^{3}H]1,25-$(OH)_2D_3$ in the presence of vehicle (C) or increasing concentrations ketoconazole (10^{-8}–10^{-4} M). (a and b) Metabolism of 0.5 nM [^{3}H]1,25-$(OH)_2D_3$. The medium of the cells was extracted and analyzed by HPLC as described in Materials and Methods. (●) [^{3}H]1,25-$(OH)_2D_3$; (○) the non-ether-extractable fraction; (▲) [^{3}H]1,24,25-$(OH)_3D_3$; (△) metabolite B; (□) metabolite C. (c) (■) Cellular accumulation of radioactivity measured as described in Materials and Methods. The parameters determined are expressed in dpm per 10^6 cells. Values are means of three cultrues with S.D. values of less than 10%.

ketoconazole concentrations resulted in a transient accumulation of 1,24,25-$(OH)_3D_3$ and two other metabolites, denoted B and C. The identity of these two metabolites, both migrating between 1,25-$(OH)_2D_3$ and 1,24,25-$(OH)_3D_3$ is not known with certainty at present (Fig. 2). However, comparison with the results obtained by others suggests strongly that these represent 24-oxo-1,25-$(OH)_2D_3$ and 24-oxo-1,23,25-$(OH)_3D_3$, respectively [12,13]. Also the rapid increase of radioac-

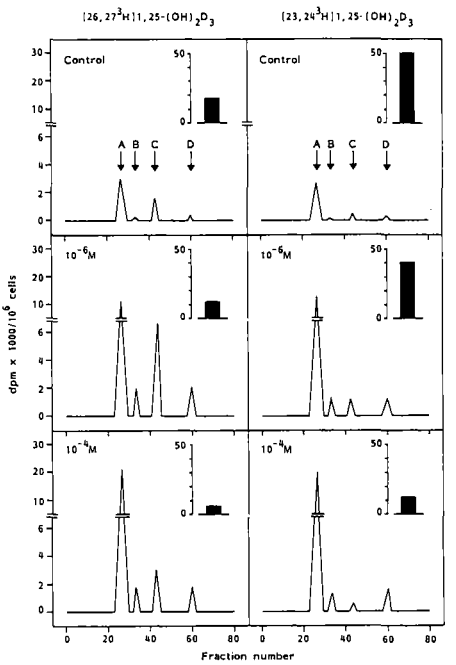

Fig. 2. Analysis by HPLC of the [^{3}H]1,25-$(OH)_2D_3$ metabolites formed at increasing ketoconazole concentrations. Cells were incubated with 0.5 nM [26,27-^{3}H]- or [23,24-^{3}H]1,25-$(OH)_2D_3$ for 4 h in the presence of vehicle or ketoconazole (10^{-6}, 10^{-4} M). The medium and cells were extracted together and chromatographed on a CPtm Spher Silica column (10×0.3 cm), eluted with hexane/isopropanol/methanol (88:10:2) at a flow rate of 0.6 ml/min. Data are expressed as dpm×1000 per 0.15 ml of eluate. Peaks A and D comigrated with 1,25-$(OH)_2D_3$ and 1,24,25-$(OH)_3D_3$, respectively. Insets, the radioactivity (dpm×1000/10^6 cells) in the non-ether-extractable fraction.

tivity in the water fraction (Fig. 1a) suggests an extensive C-24 oxidation of the [23,24-^{3}H]-1,25-$(OH)_2D_3$. In this respect, it is important to emphasize that if peaks B and C are indeed 24-oxo-1,25-$(OH)_2D_3$ and 24-oxo-1,23,25-$(OH)_3D_3$, respectively, the use of 23,24-tritiated 1,25-$(OH)_2D_3$ results in an underestimation of their real magnitude. As can be clearly seen in Fig. 2, peak C is particularly defficient in tritium when [23,24-^{3}H]1,25-$(OH)_2D_3$ was used, but was adequately labeled using [26,27-^{3}H]1,25-$(OH)_2D_3$ as the substrate. Furthermore, the loss of the tritium label in the water fraction is less marked when the label is not in the 23,24-positions, but in the 26,27-methyl groups. Therefore, metabolites B and C fit logically in the earlier described C-24 oxidation pathway [12,13]: 1,25-$(OH)_2D_3$ (A) → 1,24,25-$(OH)_3D_3$ (D) → 24-oxo-1,25-$(OH)_2D_3$ (B) → 24-oxo-1,23,25-$(OH)_3D_3$ (C) → side-chain cleavage. In this context, it is noteworthy that with increasing ketoconazole concentrations the various compounds show maximum accumulation in the reverse order, i.e., 24-oxo-1,23,25-$(OH)_3D_3$ at 10^{-6} M; 24-oxo-1,25-$(OH)_2D_3$ at $5 \cdot 10^{-6}$ M; 1,24,25-$(OH)_3D_3$ at 10^{-5} M; and 1,25-$(OH)_2D_3$ at 10^{-4} M (Fig. 1a and 1b).

Recently, we have shown that the loss of specific binding of 1,25-$(OH)_2D_3$ by intact UMR-106 cells is largely caused by metabolism of the sterol [7]. Therefore, we reasoned that inhibition of metabolism by ketoconazole might prevent the decline in specific binding. Indeed, the decrease of 1,25-$(OH)_2D_3$ metabolism caused by ketoconazole was closely associated with an increase of radioactivity in the cellular compartment (Fig. 1c). As depicted in Fig. 3, cellular radioactivity largely consisted of [^{3}H]1,25-$(OH)_2D_3$. We next examined whether the cellular accumulation of 1,25-$(OH)_2D_3$ represents specific binding. For this purpose we measured both specific cellular accumulation of [^{3}H]1,25-$(OH)_2D_3$ and receptor-bound [^{3}H]1,25-$(OH)_2D_3$ in cytosol prepared following incubation with intact cells. Previously performed Scatchard analysis and competition experiments indicated that specific cellular accumulation represents binding to the classical 1,25-$(OH)_2D_3$ receptor, showing high avidity (dissociation constant: 40 pM) and high specificity [7,14]. The time course of specific cellular accumulation and receptor-bound [^{3}H]-

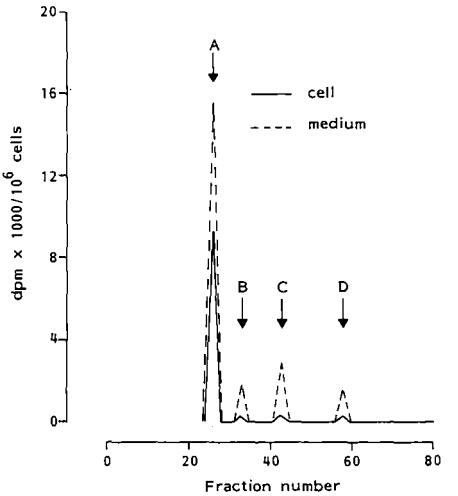

Fig. 3. Representative metabolite profiles of medium and cells after incubation of 10^6 cells for 4 h with 0.5 nM [23,24-^{3}H]1,25-$(OH)_2D_3$ in the presence of 10^{-4} M ketoconazole. Cells and medium were separately extracted and chromatographed as described in Materials and Methods.

1,25-$(OH)_2D_3$ in the presence of ketoconazole (Fig. 4) exhibited two components: an initial phase up to 2 h, which is not significantly different from control, and a second phase showing not a maintenance, but a sharp increase in both specific cellular accumulation and receptor-bound hormone. Although extraction and charcoal separation of receptor-bound [^{3}H]1,25-$(OH)_2D_3$ resulted in approx. 50% lower specific binding compared to the specific cellular accumulation, the time course for both binding parameters was identical. In addition, binding assays performed in broken cells showed that the increase in specific binding was not the result of a ketoconazole-induced change in receptor affinity (data not shown). These results indicate that the metabolism of 1,25-$(OH)_2D_3$ masks the actual available pool of specific binding sites. Therefore, the C-24 oxidation pathway for the metabolism of 1,25-$(OH)_2D_3$ seems to play an important role in controlling the intracellular levels of and, consequently, receptor occupancy by the hormone.

We and others have shown that inhibition of

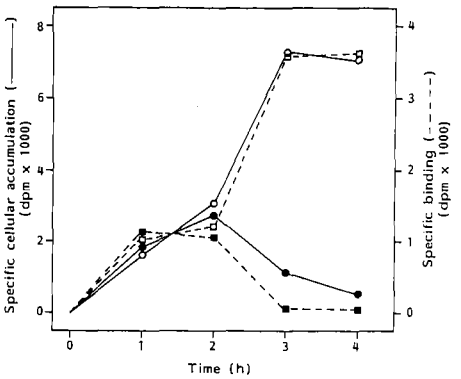

Fig. 4. The effect of ketoconazole on the time-course of [^{3}H]1,25-$(OH)_2D_3$ binding. Cells were incubated with 0.5 nM [^{3}H]1,25-$(OH)_2D_3$ without or with a 200-fold molar excess of unlabeled 1,25-$(OH)_2D_3$ in the absence or presence of 10^{-4} M ketoconazole. Cells were assayed for specific cellular accumulation and specific binding at the indicated time points. A correction for non-specific cellular accumulation and binding in the cytosol preparations was determined at each time point. The values shown represent specific cellular accumulation in the absence (●) or presence (○) of ketoconazole and specific binding in the absence (■) or presence (□) of the drug.

metabolism with the DNA transcription inhibitor actinomycine D also prevented the loss of specific binding [7,15]. However, the further increase in binding, as seen in the presence of ketoconazole, is not observed when the induction of new mRNA is blocked. Also, the 2-h lag time required before we observed a further increase in specific binding during co-incubation with ketoconazole, suggests a receptor-mediated induction event. Taken together, we interpret these findings as an indication that the 1,25-$(OH)_2D_3$-receptor appears to undergo homologous up-regulation. This phenomenon has also been reported by Costa et al. [16], although they measured receptors in cell extracts after preincubation with various vitamin D metabolites. Our results suggest that it is possible to study receptor up-regulation in intact cells if the self-induced metabolism of 1,25-$(OH)_2D_3$, which normally interferes with the measurement of receptor concentration, is prevented by compounds such as ketoconazole. This procedure also has the advantage that pretreatment of the cells with unlabeled 1,25-$(OH)_2D_3$ is unnecessary, thereby avoiding potential problems related to receptor occupancy with the hormone used in the preincubation.

In summary, we have shown that ketoconazole is a useful tool in the study of the regulation of vitamin D action at the cellular level. Because 1,25-$(OH)_2D_3$ receptor levels determine the magnitude of the mediated biological response [17], it would be expected that receptor up-regulation results in an increased responsiveness to 1,25-$(OH)_2D_3$. If ketoconazole indeed only prevents metabolism, this compound could be of value to test this hypothesis.

Acknowledgement

This study was supported by a grant from the Dutch Kidney Foundation (no. C 83.401).

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APPENDIX PAPER 4

Evidence that the self-induced metabolism of 1,25-dihydroxyvitamin D_3 limits the homologous up-regulation of its receptor in rat osteosarcoma cells

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(accepted for publication by Biochim Biophys Acta)

Summary

The osteoblast-like osteosarcoma cell line UMR-106 has been shown to possess high-affinity receptors for 1,25-dihydroxyvitamin D (1,25-$(OH)_2D_3$). Also, these cells metabolize 1,25-$(OH)_2D_3$ to more polar metabolites. As previously demonstrated (Biochim Biophys Acta 1987;175:115) the time course of specific binding of 1,25-$(OH)_2D_3$ in intact UMR-106 cells was found to be characterized by a) an ascending phase, representing association with receptor, b) a maximum at 90-120 min, and c) a rapid descending phase, closely associated with a decrease of medium 1,25-$(OH)_2D_3$ due to the metabolism of the hormone. The purpose of the present study was to further investigate the self-induced metabolism of 1,25-$(OH)_2D_3$ in relation to the homologous up-regulation of its receptor in these cells. Inhibition of metabolism of 1,25-$(OH)_2D_3$ with ketoconazole resulted, after a lag-time of about 90 min, in a sharp increase of receptor accumulation. This increase in receptor level in the presence of ketoconazole was blocked by coincubation with cycloheximide and actinomycin D. Preincubation experiments with unlabelled 1,25-$(OH)_2D_3$ showed that the elevation of hormone binding was 1,25-$(OH)_2D_3$ concentration dependent (Ed_{50} 200-300 pM). Addition of ketoconazole during these preincubations resulted in an even more pronounced accumulation of receptors, whereby the ED_{50} (50-60 pM) was comparable with the dissociation constant of the 1,25-$(OH)_2D_3$ receptor (41.3 ± 4.3 pM).

In summary these data support the concept that the self-induced metabolism of 1,25-$(OH)_2D_3$ has a dual effect: 1) *directly* by the regulation of the cellular concentration of and, consequently, receptor occupancy by the active form of vitamin D, and

2) *indirectly* by its ability to modulate the ligand-dependent regulation of the $1,25\text{-}(OH)_2D_3$.

Introduction

Recent studies have shown that the effects of $1,25\text{-}(OH)_2D_3$ are not confined to the stimulation of intestinal Ca-absorption [1] and bone resorption [2], but that the sterol also influences cell proliferation and differentiation in e.g. osteoblasts [3,4], breast carcinoma cells [5] and cells of the immune system [6,7]. Also in other tissues the finding of $1,25\text{-}(OH)_2D_3$ receptors was associated with biological effects [8,9,10]. It seems improbable that the regulation of the function of these different tissues relies solely on the synthesis of $1,25\text{-}(OH)_2D_3$ in the kidney. Possible mechanisms at the cellular level that could modulate the biological response to vitamin D are: 1) local synthesis of $1,25\text{-}(OH)_2D_3$ [11]; 2) ligand-dependent up-regulation of the $1,25\text{-}(OH)_2D_3$ receptor [12] or 3) regulation of the cellular $1,25\text{-}(OH)_2D_3$ level through the ability of the hormone to induce its own metabolism [13].

In the present study we examined both $1,25\text{-}(OH)_2D_3$ receptor up-regulation and the influence of the self-induced metabolism of the sterol on receptor occupancy. The model for study were mono-layers of the osteoblast-like osteogenic sarcoma cell, UMR-106, a cell which possesses high affinity $1,25\text{-}(OH)_2D_3$ receptors [14] and have shown an extensive metabolism of the hormone [15,16].

To determine whether $1,25\text{-}(OH)_2D_3$ receptor regulation was influenced by the self-induced metabolism of the hormone we used ketoconazole. Recent evidence obtained in our laboratory showed that this imidazole has the unique ability to inhibit $1,25\text{-}(OH)_2D_3$ metabolism in UMR-106 cells [16], without interfering with $1,25\text{-}(OH)_2D_3$ receptor accumulation. We also present data concerning the role of translational and transcriptional control of the ligand-dependent accumulation of the $1,25\text{-}(OH)_2D_3$ receptor in UMR-106 cells.

Materials and methods

The rat osteosarcoma cell line UMR-106 was supplied by Dr. M.P.M. Herrmann-Erlee (Laboratory of Cell Biology, University of Leiden, Leiden, The Netherlands). $1,25\text{-}[23,24\text{-}^3H](OH)_2D_3$ (102 Ci/mmol) was obtained from Amersham and nonradioactive hydroxylated vitamin D metabolites were generously provided by LEO Pharmaceuticals. Bovine serum albumin (BSA, fraction V), cycloheximide and actinomycin D were from Sigma, while Eagle's minimum essential medium (MEM), glutamine, penicilline and streptomycine were obtained from Flow Laboratories. Ketoconazole was obtained from Janssen Pharmaceuticals. Fetal calf serum (FCS) and the culture dishes (35 mm petri dishes and 24-well dishes) were purchased from Greiner.

Cell culture and incubation procedure

The general procedure was adapted from Sher et al [17]. UMR-106 cells were seeded at a density of 10^5 cells/cm^2 and cultured in MEM supplemented with 2 mM glutamine, 100 U/ml penicilline, 100 μg/ml streptomycine and 10% FCS. After an additional 16 h preincubation period in MEM with 2% charcoal-treated FCS [18], the confluent cultures were incubated in serum-free MEM with 0.1% BSA and the desired amount of 1,25-[^{3}H](OH)$_2$D$_3$ with or without a 200-fold molar excess of unlabelled hormone. The incubations were carried out for 0-8 h at 37°C with the culture dishes placed on a slightly angled slowly rotating plate. At the end of each incubation period the medium was removed. The cells were additionally incubated for 10 min at room temperature with Hanks' balanced salt solution containing 2% BSA and then rinsed twice more with ice-cold Hanks medium alone.

Extraction and chromatography of the medium

The medium and the Hanks' balanced salt solutions were pooled and extracted with ether. The lipid extract was subjected to HPLC on a CPtm Spher silica column (10x0.3 cm, Chrompack) with hexane-isopropanol-methanol (88:10:2) as the solvent at a flow rate of 0.6 ml/min. Radioactivity eluting with 1,25-(OH)$_2$D$_3$ and 1,24,25-(OH)$_3$D$_3$ was quantitated. The radioactivity in the non-ether extractable fraction, which represents the loss of tritium label through C24- and C23-oxidation of the 23,24-tritiated 1,25-(OH)$_2$D$_3$ [11], was also quantitated.

Whole cell binding assay

Cells were solubilized in 0.1 M NaOH, neutralized with acetic acid and counted for radioactivity. Specific cellular accumulation of 1,25-(OH)$_2$D$_3$ (SCA) was based upon the difference between the 1,25-[^{3}H](OH)$_2$D$_3$ bound to cells in the absence or presence of a 200-fold molar excess of unlabelled hormone. SCA was expressed as dpm per number of cells.

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

When cells were first preincubated with unlabelled hormone, receptor content was determined in cytosolic extracts according to the method described by Costa et al [12]. Before preparation of cell extracts, media were aspirated and the cells were incubated for an additional 2 h with MEM + 10% FCS at 37°C to promote dissociation of hormone from the receptor [12]. Afterwards, the cells were rinsed with ice-cold Hanks, trypsinized and, after inactivation of trypsine, centrifuged. The 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 200 uml aliquots were incubated at 0°C overnight with 1 nM 1,25-[^{3}H](OH)$_2$D$_3$ in the presence or absence of a 200-fold molar excess of unlabelled hormone. Receptor bound 1,25-(OH)$_2$D$_3$ was separated from unbound sterol by charcoal adsorption [19]. Protein concentration was measured according to the method of Bradford [20].

Results

In Fig. 1 the time-course of cellular accumulation and metabolism of 1,25-[^{3}H](OH)$_2$D$_3$ is shown. As can be seen there is a rapid increase in specific cellular accumulation (SCA) to a maximum at 90-120 min, followed by a rapid decrease. Although at lower concentrations of 1,25-[^{3}H](OH)$_2$D$_3$ the increase of SCA was more gradual, this did not influence the time point at which maximum SCA was achieved (not shown.)

The subsequent decrease of SCA was closely associated with the decline of 1,25-[^{3}H](OH)$_2$D$_3$ concentration in the medium (Fig. 1). This was accompanied by a transient appearance of 1,24,25-[^{3}H](OH)$_3$D$_3$, although most 1,25-[^{3}H](OH)$_2$D$_3$ was rapidly further converted to non-ether extractable products. Recently, we and others [21] have demonstrated that 1,25-(OH)$_2$D$_3$ is metabolized in UMR-106 cells along the C24-oxidation pathway with 1,24,25-(OH)$_3$D$_3$, 24-oxo-1,25-(OH)$_2$D$_3$ and 24-oxo-1,23,25-(OH)$_3$D$_3$ as intermediates [16]. Side chain cleavage is the final step in this pathway possibly with the formation of calcitroic acid. Therefore, it is important to emphasize that if 1,25-[23,24-^{3}H](OH)$_2$D$_3$ is used the loss of radioactivity as ^{3}H$_2$O is an overall estimate of 1,25-(OH)$_2$D$_3$ metabolism.

Fig. 2 shows the saturation curve for SCA data from 2 h incubations. Scatchard analysis was compatible with a single class of binding sites. The apparent dissociation constant (K_d) calculated from the slope of the regression line was approximately 40 pM. The intercept of the curve with the abscissa yielded a maximum number of binding sites of 8 fmol/well (20-30 fmol/mg protein). Competitive binding experiments revealed the binder to prefer 1,25-(OH)$_2$D$_3$ > 1,24,25-(OH)$_3$D$_3$ ≫ 25-(OH)D$_3$ > 24,25-(OH)$_2$D$_3$ (data not shown).

To further investigate the influence of 1,25-(OH)$_2$D$_3$ metabolism on SCA we used different inhibitors of the self-induced metabolism of the hormone. As inhibitor of the cytochrome P-450 dependent 24-hydroxylase enzyme we used ketoconazole. Recently, we found that this imidazole caused a dose-dependent inhibition of the C24-oxidation of 1,25-(OH)$_2$D$_3$ metabolism [16]. At 10^{-4} M ketoconazole, a concentration that did not affect the viability of the cells as determined by trypan blue exclusion, we observed an approximately 70% decrease of metabolism. Furthermore, we tested the influence of cycloheximide and actinomycin D, both well-known inhibitors of the receptor-mediated induction of 1,25-(OH)$_2$D$_3$ metabolism, on the time-course of SCA. At the doses used not obvious toxic effects were noted and trypan blue exclusion was unaffected. In fig. 3 the effects of the different inhibitors on SCA are shown. In the presence of ketoconazole the time-course of SCA exhibited two components: an initial phase up to about 90 min, which is not significantly different from control, and a second phase showing a sharp increase of SCA instead of the decline observed without ketoconazole. On the other hand, incubation with cycloheximide resulted within 1 h in a significant decrease of the maximum SCA achieved, while

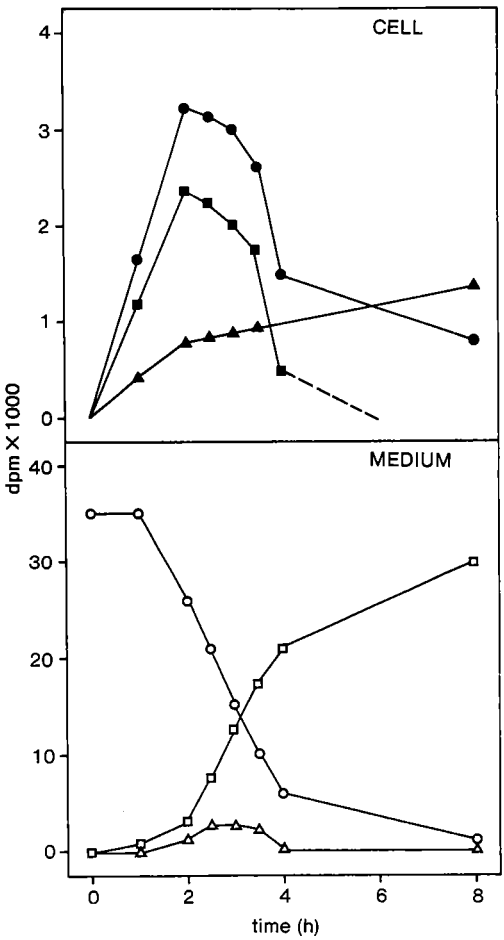

Figure 1: A typical time course of cellular accumulation and metabolism of 1,25-$(OH)_2D_3$ in UMR-106 cells. Confluent cultures (35 mm dishes) were incubated with 0.5 nM 1,25-[23,24-^{3}H]$(OH)_2D_3$ alone or in the presence of a 200-fold molar excess of unlabelled 1,25-$(OH)_2D_3$. ***Upper panel:*** *Cellular accumulation of 1,25-[^{3}H]$(OH)_2D_3$, measured as described in Materials and Methods. (●) cellular accumulation of 0.5 nM 1,25-[^{3}H]$(OH)_2D_3$; (▲) cellular accumulation of 0.5 nM 1,25-[^{3}H]$(OH)_2D_3$ + a 200-fold molar excess of unlabelled 1,25-$(OH)_2D_3$; (■) specific cellular accumulation of 1,25-[^{3}H]$(OH)_2D_3$.* ***Lower panel:*** *Metabolism of 0.5 nM 1,25-[^{3}H]$(OH)_2D_3$. The medium of the cells was extracted and analysed by HPLC as described in Materials and Methods. (○)1,25-[^{3}H]$(OH)_2D_3$; (△)1,24,25-[^{3}H]$(OH)_3D_3$; (□) the non-ether-extractable fraction. The parameters measured are expressed in dpm per well. Each well contained approximately 2.10^6 cells. Values are means of 3 cultures with SD values less than 10%.*

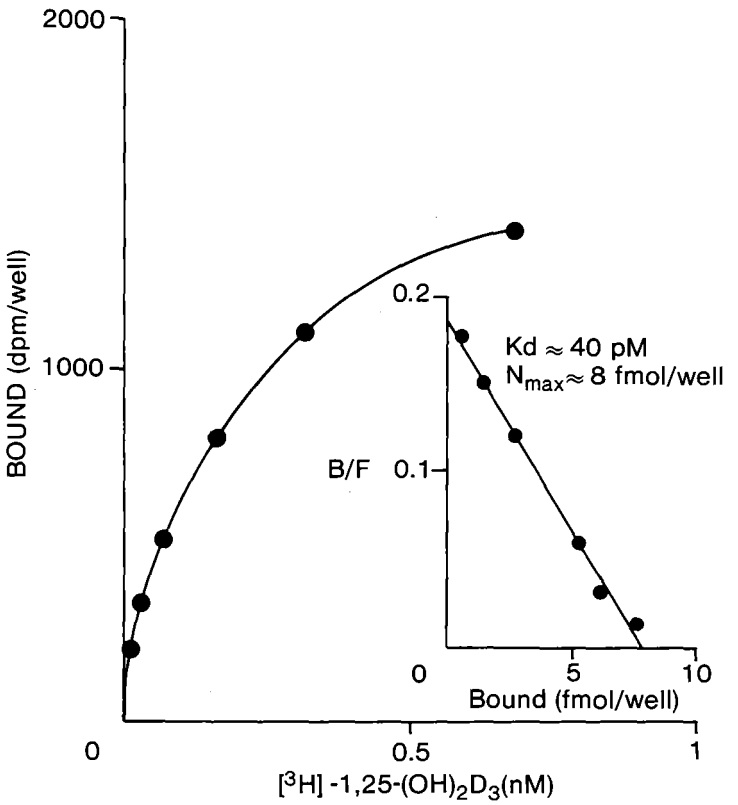

Figure 2: Saturation and Scatchard analysis of specific cellular accumulation of 12,5-$(OH)_2D_3$. Confluent cultures (24-well dishes) were incubated for 2 h with varying concentrations of 1,25-[^{3}H]$(OH)_2D_3$ with or without a 200-fold excess of unlabelled 1,25-$(OH)_2D_3$. Each well contained approximately 10^6 cells. The mean ± SEM of 3 experiments yields an apparent affinity (Kd) of 41.3 ± 4.3 pM and a maximum number of binding sites (Nmax) of 9.8 ± 1.1 fmol/well.

actinomycin D did not affect binding until 2 h. Both compounds prevented the normally found loss of SCA for up to 4 h. Scatchard analyses performed at 2 h showed that both cycloheximide and actinomycin D did not influence the apparent affinity of the 1,25-$(OH)_2D_3$ receptor, while cycloheximide but not actinomycin D greatly reduced the maximum binding capacity (data not shown).

To ascertain whether coincubation with cycloheximide and actinomycin D, respectively, resulted in an inhibition of 1,25-[^{3}H]$(OH)_2D_3$ metabolism the media were further analysed (Fig. 4). In contrast to the effect of actinomycin D the inhibition of

metabolism with cycloheximide was only partial, which resulted in a delayed, but still significant accumulation of polar metabolites. Concentrations up to 10 μg cycloheximide per ml were not more effective.

To determine the role of the novo protein synthesis and induction of new mRNA in the observed increase in receptors in the presence of ketoconazole, we examined the ability of cycloheximide and actinomycin D, respectively, to modulate this event. As can be seen in Table I both cycloheximide and actinomycin D blocked the increase in receptor level as found when metabolism was inhibited with ketoconazole. The maximum SCA achieved in the presence of both cytotoxic drugs was not influenced by the addition of ketoconazole. In this respect the results are identical to those shown in Fig. 3.

The effect of hormone concentration was evaluated by preincubating the cells with increasing concentrations of unlabelled 1,25-$(OH)_2D_3$ in the absence or presence of ketoconazole. Before receptor concentration was assayed in cytosolic extracts the mono-layers were additionally incubated for 2 h with medium containing 10% FCS to absorb 1,25-$(OH)_2D_3$ dissociated from its receptor [12]. As shown in Fig. 5

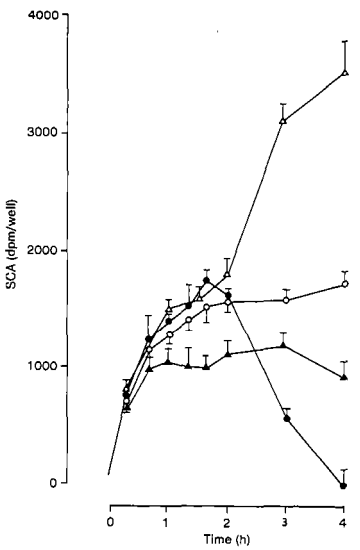

Figure 3: A typical time course of the effect of ketoconazole, cycloheximide and actinomycin D on specific cellular accumulation (SCA) of 1,25-$(OH)_2D_3$ in UMR-106 cells. Confluent cultures (24-well dishes) were incubated with 0.5 nM 1,25-[^{3}H]$(OH)_2D_3$ (with or without a 200-fold molar excess of unlabelled 1,25-$(OH)_2D_3$) alone or in the presence of ketoconazole (10^{-4} M), cycloheximide (2 μg/ml) or actinomycin D (1 μg/ml), respectively. SCA was measured at the times indicated. Each well contained approximately 10^6 cells. Values are means ± SD of 3 cultures. Control (●); ketoconazole (△); cycloheximide (▲); actinomycin D (○).

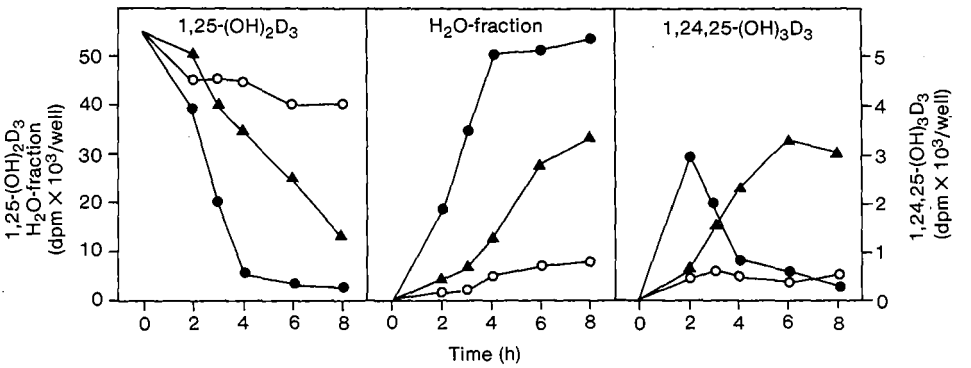

Figure 4: Effect of cycloheximide and actinomycine D on 1,25-$(OH)_2D_3$ metabolism. Confluent cultures (35 mm dishes) were incubated with 0.5 nM 1,25-[^{3}H]$(OH)_2D_3$ alone or in the presence of cycloheximide (2 μg/ml) or actinomycine D (1 μg/ml). The medium and cells were extracted and analysed by HPLC as described in Materials and Methods. Each well contained approximately 2.10^6 cells. Values are means of 4 cultures with SD values less than 10%. Control (●); cycloheximide (▲); actinomycin D (○).

pretreatment for 4 h with various concentrations 1,25-$(OH)_2D_3$ resulted in a dose-dependent increase of receptors. As little as 10^{-11} M 1,25-$(OH)_2D_3$ resulted in a significant rise of receptor concentration, while the maximum effect was reached at 10^{-8} M. Inhibition of metabolism during the preincubation period resulted in a significant shift of the dose-response curve to the left without an influence on the maximum receptor concentration reached. The approximate ED_{50} in the presence of

Table I: Influence of ketoconazole, cycloheximide and actinomycin D on the maximum SCA achieved.

Conditions	**SCA (% of control)**
Control	100
Ketoconazole	215.3 ± 13.5*
Cycloheximide	61.5 ± 5.8*
Cycloheximide + Ketoconazole	52.1 ± 9.6*
Actinomycin D	88.5 ± 14.2
Actinomycin D + Ketoconazole	101.9 ± 3.8

Confluent cultures (24-well dishes) were incubated with 0.5 nM 1,25-[^{3}H]$(OH)_2D_3$ alone or in the presence of vehicle (control), ketoconazole (10^{-4} M), cycloheximide (2 ug/ml), actinomycin D (1 ug/ml) or a combination of these compounds. Maximum SCA in control cultures was measured at 90 min, while SCA during coincubation with the compounds was assayed after 4 h. Results are expressed as a percentage of control (= 100%). *$p < 0.01$ difference from control.

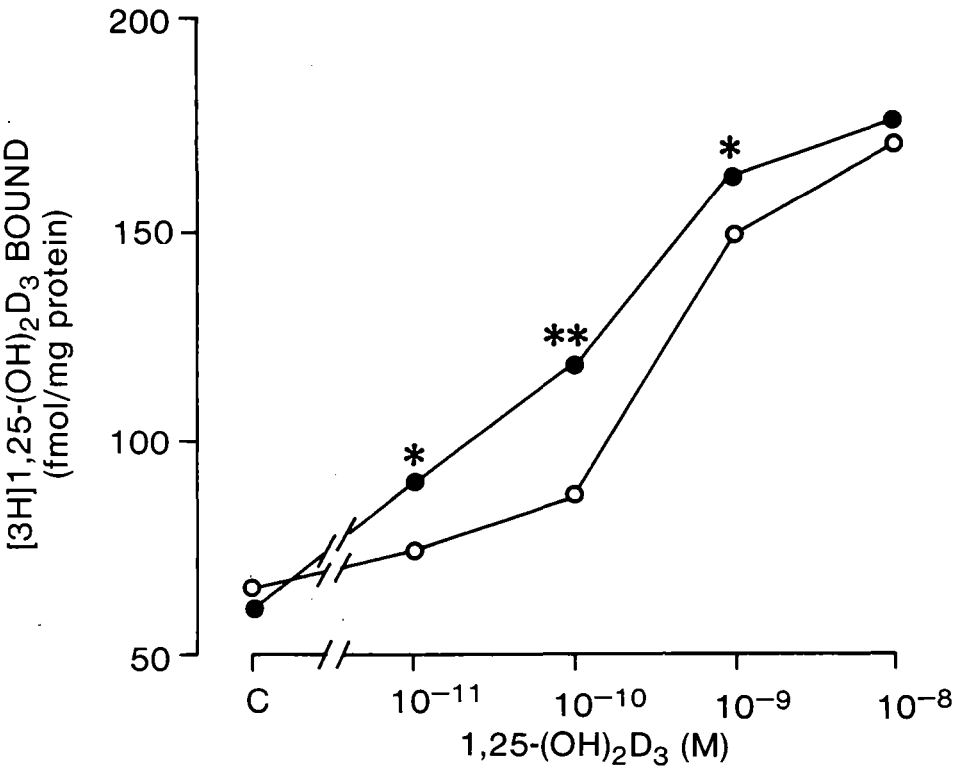

*Figure 5: 1,25-$(OH)_2D_3$ receptor up-regulation measured after preincubation with various concentrations unlabelled 1,25-$(OH)_2D_3$ with or without 10^{-4}M ketocanazole. After 4 h preincubation hormone was removed and cells were incubated for an additional 2 h with MEM + 10% FCS. 1,25-$(OH)_2D_3$ receptor concentration was assayed in triplicate after extraction of the cells with hypertonic buffer as described in Materials and Methods. Control (○); Ketoconazole (●). *$p<0.05$; *$p<0.01$ difference from control.*

ketoconazole was 40-60 pM, five fold lower than the ED_{50} in the absence of the imidazole. Finally, addition of actinomycin D (1 μg/ml) during the preincubation with radioinert 1,25-$(OH)_2D_3$ resulted in a similar inhibition of receptor accumulation as found in the presented whole cell binding experiments (results not shown).

Discussion

The data we have presented suggest that 1,25-$(OH)_2D_3$ is capable to induce an up-regulation of its own receptor. A similar phenomenon has been described for other osteosarcoma cell lines [22] and cloned kidney cells [12]. Furthermore, evidence is provided to indicate that the self-induced metabolism of the hormone plays a

regulatory role in the homologous up-regulation of the 1,25-$(OH)_2D_3$ receptor. Therefore, cellular responses to the active form of vitamin D appears to be regulated by mechanisms in addition to simple hormone-receptor interaction.

The Scatchard analysis data and the competitive binding experiments support the view that SCA represents a class of binding sites with the characteristics of the classical 1,25-$(OH)_2D_3$ receptor. The apparent dissociation constant derived in this manner is very similar to those found in the cytosols prepared from disrupted cells [14]. From earlier experiments [11] we have learned that rotation of the wells on a slightly angled, slowly rotating plate enhanced metabolism considerably. Using this method we show that is is also possible to obtain a maximum SCA at a time point comparable to that found for equilibrium of 1,25-$(OH)_2D_3$ in cytosolic preparations of these cells [14].

The parallelism between the loss of SCA and the decrease of 1,25-$(OH)_2D_3$ concentration in the medium, due to the ability of the hormone to induce its own metabolism, suggests that both processes are linked. It was not the purpose of this study to unequivocally identify the more polar metabolites formed. However, previous data support and extensive C24-oxidation along the pathway: 1,25-$(OH)_2D_3$ → 1,24,25-$(OH)_3D_3$ → 24-oxo-1,25-$(OH)_2D_3$ → 24-oxo-1,23,25-$(OH)_3D_3$ → side chain cleavage [16,21]. We have documented a similar pathway for 25-$(OH)_2D_3$ in UMR-106 cells after induction of metabolism with 1,25-$(OH)_2D_3$ [15]. The effect of cycloheximide and actinomycin D in our system once again demonstrates that the induction of 1,25-$(OH)_2D_3$ of its own metabolism is a receptor-mediated event and involves de novo protein synthesis.

An important difference between our results and those using other cell lines [22,23] appears to be the rapid metabolism of 1,25-$(OH)_2D_3$ in UMR-106 cells. However, inhibition of metabolism with ketoconazole resulted in a time-course of SCA comparable to that recently in ROS 17/2 cells [22], which suggests that the catabolism of the hormone in UMR-106 cells masks the actual available pool of receptors. Therefore, the rapidity of 1,25-$(OH)_2D_3$ metabolism in our system and the ability of ketoconazole to inhibit this metabolism provides a system to study the influence of the self-induced metabolism of the sterol on receptor regulation.

The preincubation experiments with radioinert 1,25-$(OH)_2D_3$ indicates a homologous up-regulation of the 1,25-$(OH)_2D_3$ receptor and show that this up-regulation is a 1,25-$(OH)_2D_3$ concentration dependent phenomenon. Addition of ketoconazole results in an even more pronounced accumulation of receptors. In this respect it is interesting that the ED_{50} found in the presence of ketoconazole is comparable to the Kd of the receptor, while in the absence of the imidazole a 5-fold higher ED_{50} was observed. This change in the apparent ED_{50} indicates that cellular metabolism determines the amount of 1,25-$(OH)_2D_3$ ultimately available for the ligand-dependent accumulation of the receptor. That at higher 1,25-$(OH)_2D_3$ concen-

trations, ketoconazole had no additional effect could be explained by the fact that despite metabolism in the control cultures enough hormone remains to induce a maximal rise in receptor level. On the other hand, receptor occupancy by unlabelled hormone from the preincubation could result in an erroneous decrease in receptor number [12].

Treatment of T 47D breast cancer cells with 1,25-$(OH)_2D_3$ has been shown to decrease 1,25-$(OH)_2D_3$ receptor binding [23]. Although the hormone induces its own metabolism in these cells, binding cannot be restored by the addition of fresh hormone. This phenomenon has been referred to as 'receptor processing' and is thought to be due to a loss of hormone binding activity after ligand-receptor action in the nucleus. In our study the enhanced accumulation of receptors when the medium 1,25-$(OH)_2D_3$ concentration was kept relatively constant by inhibiting the metabolism with ketoconazole does not support an important role for receptor processing in UMR-106 cells under our experimental conditions. Also the lack of evidence for such an event in other cell lines [12,22], including breast cancer cells (MCF-7) [12], suggests that 'receptor processing' as described by Sher et al [21] might be restricted to T 47D cells.

General inhibitors of protein and RNA synthesis were employed to determine whether receptor accumulation was under translational and/or transcriptional control. The maximum SCA achieved in the presence of cycloheximide was significantly lower than that obtained in control cultures, whereas actinomycin D does not produce a significant alteration in the maximum SCA at 2 h (Fig. 3). In other studies with 1,25-$(OH)_2D_3$-depleted UMR-106 cells incubation with cycloheximide led to a loss of receptor assayable in cytosol preparations with a half-time of 2-3 h (data not shown). This suggests that maintenance of unoccupied receptor levels does depend upon continuous new protein synthesis an provides and explanation for the observed effect of the protein synthesis inhibitor on the maximum SCA achieved.

We interpret the inhibition of receptor accumulation by actinomycin D to indicate that receptor up-regulation may be dependent on new RNA synthesis. However, this observation does not necessarily mean that receptor accumulation, as measured in the whole cell binding studies, is only dependent on 1,25$(OH)_2D_3$ receptor-mediated mechanism. A previous report by Hirst and Feldman [24] indicates that introduction of a fresh and 1,25-$(OH)_2D_3$ depleted medium induces a rise in receptor level within 2 h. Preliminary results in our laboratory also showed that UMR-106 cells exhibited a rapid and actinomycin D sensitive increase of cellular receptor content in response to a medium change (manuscript in preparation). Therefore, we can not exclude that a part of the up-regulation observed in our whole cell binding experiments results from the medium change at the start of the experiment. Nevertheless, it is clear that the major component of receptor accumulation is induced by 1,25-$(OH)_2D_3$ independent of a medium change (Fig. 5). That treatment of 3T6 fibroblasts with

1,25-$(OH)_2D_3$ leads to an increase in the apparent concentration of mRNA for its receptor support this view [25].

Recently, Costa et al [26] provided evidence that receptor up-regulation after exposure of cells to 1,25-$(OH)_2D_3$ also results from a prolongation of receptor half-life in addition to an increase in receptor synthesis. The finding that SCA remains constant between 1.5 and 4 h in the presence of cycloheximide and actinomycin D in our system points in the same direction.

Taken together, our findings indicate that the self-induced metabolism of 1,25-$(OH)_2D_3$ in osteoblast-like cells limits the response of these cells to the hormone by restricting the intracellular availability of 1,25-$(OH)_2D_3$ and, consequently, the 1,25-$(OH)_2D_3$-dependent up-regulation of its receptor. In other words, the self-induced metabolism provides a mechanism that could counteract an upward spiral of homologous receptor up-regulation and, therefore, may represent a negative feedback mechanism against further receptor up-regulation at the cellular level.

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Summary

It is well known that the biologically most active metabolite of vitamin D, 1,25-$(OH)_2D_3$, is an essential hormone in the regulation of calcium homeostasis. Furthermore, evidence has accumulated to suggest that 1,25-$(OH)_2D_3$ regulates bone metabolism not only indirectly via its effect on intestinal absorption of calcium and phosphorus, but also directly through actions on bone remodelling cells and their progenitors. The aim of the present thesis has been to contribute to a better understanding of the effects of 1,25-$(OH)_2D_3$ on the osteoblastic population and to investigate the cellular mechanisms involved in the regulation of the biological response to the hormone (Chapter 2).

In Chapter 1, the current knowledge with respect to the metabolism, the mechanism of action and the biological role of vitamin D is reviewed. The classical role of vitamin D as an important regulator of calcium homeostasis is discussed but also the recently obtained evidence, suggesting a role for 1,25-$(OH)_2D_3$ in the differentiation of cells belonging to both the 'classical' (i.e. bone) and 'non-classical' target tissues (especially the immune system) of vitamin D. The data from the literature also indicate that the mode of action of vitamin D is similar to that of steroid and thyroid hormones, with the active metabolite, 1,25-$(OH)_2D_3$, complexing with a selective, high-affinity binding protein (receptor) that concentrates the hormone in the nucleus. The occupied receptor is then postulated to regulate gene expression and induce proteins that alter the functions of target cells. However, recent reports also suggest non-receptor mediated effects of 1,25-$(OH)_2D_3$. With respect to vitamin D metabolism it is important to note that 1,25-$(OH)_2D_3$ is the main inducer and regulator of the enzyme 24-hydroxylase in a wide variety of target tissues. In other words, 1,25-$(OH)_2D_3$ seems to be capable to induce its own metabolism.

The first line of study concerned the investigation of the effect of 1,25-$(OH)_2D_3$ on growth and maturation (differentiation) of osteoblast-like (OB) cells (Chapter 3, section 3.3.1 and appendix paper 1). As a parameter of maturation alkaline phosphatase (AP) activity was used. We found that 1,25-$(OH)_2D_3$ induces a dose-dependent increase of AP activity in both primary cultures of foetal rat (ROB) and of human OB cells. By changing cell density and culture conditions evidence was obtained to suggest that the sterol modulates proliferation and prevents the dedifferentiation of osteoblasts in culture. In addition, it appears that for the anti-proliferative effect of 1,25-$(OH)_2D_3$ cell-cell interactions are essential, because this phenomenon was only observed in densely seeded cultures of both ROB cells and rat osteosarcoma

cells (UMR-106). Finally, the fact that the action of 1,25-$(OH)_2D_3$ on AP is evident under conditions where no inhibition of proliferation is found suggests that the effect of the hormone on AP activity is not linked to its growth-inhibiting properties.

Under comparable experimental conditions also the effect of 1,25-$(OH)_2D_3$ on the adenylate cyclase system was studied (Chapter 3, section 3.3.2 and appendix paper 1). Treatment of ROB cells with 1,25-$(OH)_2D_3$ resulted in an attenuation of the cAMP response to PTH, without an effect on the EC_{50} of PTH. Although an action of 1,25-$(OH)_2D_3$ on the number of PTH receptors can not be excluded, additional experiments with forskolin suggest that 1,25-$(OH)_2D_3$ also interferes with the adenylate cyclase system, either at the level of the regulatory unit or on the interaction of the regulatory with the catalytic unit. In accordance with this site of action is the observation that in UMR-106 cells 1,25-$(OH)_2D_3$ also attenuates the cAMP response to the β-agonist isoproterenol. In contrast, in rapidly growing ROB cell cultures an opposite effect of the sterol on the isoproterenol-stimulated cAMP accumulation was found. Whether this increased cAMP response may be related to the heterogenous character of primary cultures and, therefore, may indicate different stages of osteoblastic maturation remains to be established.

In the last section (3.3.3) of chapter 3 we demonstrate that foetal chicken OB (COB) cells can synthesize 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$. Only two factors could be identified which influenced the accumulation of both dihydroxyvitamin D_3 derivatives: (1) vitamin D binding protein(s) and (2) the ability of 1,25-$(OH)_2D_3$ to induce the enzyme 24-hydroxylase (resulting in a rapid accumulation of 24,25-$(OH)_2D_3$ and a rapid decrease of the initially formed 1,25-$(OH)_2D_3$). Other factors, like calcium and PTH, appeared to have no influence on this extrarenal synthesis of 1,25-$(OH)_2D_3$. Whether 1,25-$(OH)_2D_3$ produced by COB cells represents a paracrine factor, for instance in the recruitment of osteoclasts, awaits further study. The absence of a measurable 1α-hydroxylase activity in ROB cells (Chapter 4) may indicate that our findings in COB cells are species dependent.

Is 1,25-$(OH)_2D_3$ production by OB cells one of the possible mechanism to regulate vitamin D action at the cellular level, the self-induced metabolism of the hormone and regulation of its receptor number are two other possibilities we have investigated (Chapter 4, appendix paper 3 and 4). In rat bone cells evidence was obtained for the presence of a 1,25-$(OH)_2D_3$-inducible side chain oxidation of the hormone along the following metabolic pathway: 1,25-$(OH)_2D_3$ $\rightarrow$ 1,24,25-$(OH)_3D_3$ $\rightarrow$ 24-oxo-1,25-$(OH)_2D_3$ 24-oxo-1,23,25-$(OH)_3D_3$ $\rightarrow$ side chain cleavage. A similar pathway was identified for 25-(OH)D_3. Subsequently, a system was developed to measure simultaneously 1,25-$(OH)_2D_3$ receptor binding and metabolism in intact osteoblast-like osteosarcoma cells (UMR-106). We found that the time-course of specific binding in UMR-106 cells was characterized by (a) an ascending phase, representing association of the hormone with its receptor, (b) a maximum binding at 90-120 min,

and (c) a rapidly descending phase closely associated with a decrease of the medium 1,25-$(OH)_2D_3$ concentration due to side chain metabolism of the hormone. Inhibition of metabolism with ketoconazole not only prevented the descending phase, but resulted after a lag-time of about 90 min in a sharp increase of receptor accumulation. This so-called '1,25-$(OH)_2D_3$ receptor up-regulation' appears to be ligand-dependent and superimposed on an up-regulation caused by a not yet identified factor related to the medium change at the start of the experiments. That actinomycin D prevented the observed increased of hormone binding suggests the involvement of new mRNA synthesis in the up-regulation of the 1,25-$(OH)_2D_3$ receptor. On the other hand, we also obtained some evidence that may indicate a decreased degradation rate of the receptor by hormone. Therefore, it seems likely that the homologous up-regulation of the 1,25-$(OH)_2D_3$ receptor results from both an enhancement of its synthesis rate and a prolongation of receptor half-life. Furthermore, the data presented in appendix paper 4 support the concept that the self-induced metabolism of 1,25-$(OH)_2D_3$ has a dual effect: (1) *directly* by the regulation of the cellular concentration of and, consequently, receptor occupancy by the active form of vitamin D and (2) *indirectly* by its ability to modulate the ligand-dependent regulation of the 1,25-$(OH)_2D_3$ receptor.

Taken together, these results indicate that the ligand-dependent regulation of cellular receptor levels provides a mechanism for enhancing the sensitivity of a target tissue to relatively small fluctuations in circulating 1,25-$(OH)_2D_3$ levels by amplifying the primary hormonal signal. On the other hand, the higher rate of catabolism of the hormone, observed at higher 1,25-$(OH)_2D_3$ receptor levels (Chapter 4, section 4.3.3), facilitates a rapid and efficient decrease of the cellular response to 1,25-$(OH)_2D_3$ and, consequently, could counteract an upward spiral of homologous receptor up-regulation.

In Chapter 5 the results of a relatively short-term treatment with 1α-$(OH)D_3$ (3 months) on bone turnover and mineralization in patients with predialysis renal osteodystrophy are presented. Twenty-nine patients with a creatine clearance of 17.6 ± 1.7 ml/min were treated with 1α-$(OH)D_3$ (average daily dose 0.55 μg). At the start of the study 14 patients had an osteoblast seams length (Ob.Pm) of less that 4% (group I) and high osteoid parameters, whereas 13 patients (group II) with an osteoblast seams length greater than 4% also had clear histological signs of hyperparathyroidism, including higher iPTH values. Treatment with 1α-$(OH)D_3$ resulted in a substantial suppression of secondary hyperparathyroidism in group II, with a fall in both Ob.Pm and the cancellous bone perimeter occupied by tetracycline double label. On the other hand, no change of the mineral appositional rate was observed, suggesting that treatment with 1α-$(OH)D_3$ reduces bone matrix production through a decline of the number of Basis Multicellular Units. In Group II, treatment also resulted in the development of a positive correlation between Ob.Pm and the

number of osteoclasts. This finding could be compatible with a role for 1,25-$(OH)_2D_3$ in the coupling between osteoblasts and osteoclasts. Finally, when calculated for both groups together, before and after treatment serum calcium was negatively correlated with osteoid seams length, while a positive correlation was found with the (thionine) mineralization front expressed as a percentage of the osteoid seams. Therefore, the treatment-induced increase of the mineralization front may not be dependent on a direct effect of 1,25-$(OH)_2D_3$ on the osteoblasts, but in contrast seems to be related to the ability of 1α-$(OH)D_3$ to increase serum calcium levels by enhancing intestinal calcium absorption.

Samenvatting

Het is bekend dat de biologisch meest actieve metaboliet van vitamine D, 1,25-$(OH)_2D_3$, een belangrijk hormoon is bij de regulatie van de calcium-homeostase. Bovendien zijn er aanwijzingen die suggeren dat de invloed van 1,25-$(OH)_2D_3$ op het skelet niet alleen bepaald wordt door het (indirecte) effect van het hormoon op de intestinale opname van calcium en fosfaat, maar ook door een directe invloed op de differentiatie en de activiteit van cellen betrokken bij de botombouw. Het doel van dit proefschrift is een bijdrage te leveren aan een beter begrip van de effecten van 1,25-$(OH)_2D_3$ op osteoblasten en voorts de cellulaire mechanismen te bestuderen die betrokken zijn bij de regulatie van de uiteindelijke biologische respons op het hormoon (Hoofdstuk 2).

In Hoofdstuk 1 wordt een overzicht gegeven van de huidige kennis betreffende het metabolisme, het werkingsmechanisme en de biologische rol van vitamine D. De centrale plaats van vitamine D in de calcium-homeostase wordt besproken, maar ook wordt ingegaan op de rol van 1,25-$(OH)_2D_3$ bij de differentiatie van cellen die behoren tot zowel de 'klassieke' (b.v. bot) als de 'niet klassieke' doelwit-organen (b.v. het immuun-systeem). De gegevens uit de literatuur tonen ook aan dat het werkingsmechanisme van vitamine D overeenkomt met dat beschreven voor andere steroïde hormonen. De actieve metaboliet, 1,25-$(OH)_2D_3$, bindt zich met hoge affiniteit aan een specifieke receptor. Interactie van dit receptor-ligand complex met het genoom leidt tot transcriptie van DNA in RNA waarna via translatie eiwitten worden gesynthetiseerd die uiteindelijk de specifieke effecten van 1,25-$(OH)_2D_3$ op de cel bepalen. Er zijn echter ook aanwijzingen voor het bestaan van niet via de receptor gemedieerde effecten van 1,25-$(OH)_2D_3$. Met betrekking tot het metabolisme van vitamine D is het belangrijk vast te stellen dat 1,25-$(OH)_2D_3$ in een groot aantal van zijn doelwit-organen het enzym 24-hydroxylase induceert en reguleert. Met andere woorden: 1,25-$(OH)_2D_3$ lijkt in staat zijn eigen metabolisme te induceren.

Het eerste onderdeel van de studie betrof het onderzoek naar de effecten van 1,25-$(OH)_2D_3$ op de groei en rijping (differentiatie) van osteoblast-achtige (OB) cellen (Hoofdstuk 3, sectie 3.3.1 en appendix publikatie 1). Het enzym alkalisch fosfatase (AP) werd hierbij gebruikt als maat voor de maturatie. We vonden dat 1,25-$(OH)_2D_3$ een dosis-afhankelijke stimulatie induceerde van de AP activiteit in zowel ratte- (ROB) als humane OB cellen. Door veranderingen aan te brengen in de celdichtheid en de kweekcondities werden aanwijzingen verkregen dat 1,25-$(OH)_2D_3$ in ROB cultures de proliferatie moduleert en de dedifferentiatie van de cellen

tegengaat. Het anti-proliferatieve effect van het sterol werd alleen in dicht uitgezaaide kweken gevonden (zowel ROB als ratte-osteosarcoom cellen), hetgeen er op wijst dat cel-cel contact voor dit effect van belang is. Dat ook een stijging van de AP activiteit werd gevonden onder omstandigheden waarbij 1,25-$(OH)_2D_3$ geen remming van de proliferatie gaf suggereert dat dit effect van het hormoon niet strikt gebonden is aan zijn celgroei-remmende eigenschappen.

Onder vergelijkbare omstandigheden werd ook het effect van 1,25-$(OH)_2D_3$ op het adenylaat-cyclase systeem bestudeerd (Hoofdstuk 3, sectie 3.3.3 en appendix publikatie 1). Behandeling van ROB cellen met 1,25-$(OH)_2D_3$ resulteerde in een verlaging van de cAMP-response op PTH, zonder een invloed op de EC_{50} voor PTH. Hoewel een invloed van 1,25-$(OH)_2D_3$ op het aantal PTH-receptoren niet kan worden uitgesloten, toonden additionele experimenten met forskoline aan dat 1,25-$(OH)_2D_3$ ook een invloed heeft op het adenylaat-cyclase systeem ter hoogte van het koppelingseiwit of van de interactie van dit eiwit met de katalytische 'unit'. In overeenstemming met een dergelijke invloed van 1,25-$(OH)_2D_3$ is de waarneming dat in osteosarcoom cellen de cAMP response op isoproterenol ook wordt geremd. Anderzijds werd in snel groeiende ROB cultures juist een verhoging van de door isoproterenol gestimuleerde cAMP accumulatie gevonden. In hoeverre dit effect gerelateerd is aan de vrij heterogene celpopulatie in ROB-cultures en dientengevolge een uiting zou kunnen zijn van het bestaan van verschillende differentiatie stadia van de osteoblast is op dit moment niet te beantwoorden.

In het laatste onderdeel (sectie 3.3.3) van hoofdstuk 3 wordt aangetoond dat foetale kippe-OB-(COB)-cellen in staat zijn 1,25-$(OH)_2D_3$ en 24,25-$(OH)_2D_3$ te synthetiseren. Hierbij wordt de mate van accumulatie van beide dihydroxyvitamine D-derivaten voornamelijk bepaald door de aanwezigheid van vitamine D-bindende eiwit(ten) en de mogelijkheid van 1,25-$(OH)_2D_3$ om het enzym 24-hydroxylase te induceren. Andere factoren, zoals calcium en PTH, lijken geen invloed te hebben. In hoeverre lokaal in het bot gevormd 1,25-$(OH)_2D_3$ werkzaam is als paracriene factor, b.v. in de koppeling tussen osteoblast en osteoclast, dient nader te worden onderzocht. De afwezigheid van een meetbare 1α-hydroxylase-activiteit in ROB cellen (Hoofdstuk 4) zou er op kunnen wijzen dat bovengenoemde extrarenale 1,25-$(OH)_2D_3$-synthese species-afhankelijk is.

Vormt de produktie van 1,25-$(OH)_2D_3$ door OB cellen één van de mogelijkheden om de vitamine D activiteit op cellulair niveau te reguleren, het metabolisme van 1,25-$(OH)_2D_3$ en de regulatie van het aantal receptoren voor het hormoon zijn twee andere opties die werden bestudeerd (Hoofdstuk 4, appendix publicaties 3 en 4). In osteoblasten van verschillende herkomst werd aangetoond dat 1,25-$(OH)_2D_3$ zijn eigen katabolisme reguleert via een oxidatie van de zijketen (1,25-$(OH)_2D_3$ → 1,24,25-$(OH)_3D_3$ → 24-oxo-1,25-$(OH)_2D_3$ → 24-oxo-1,23,25-$(OH)_3D_3$ → klieving van de zijketen). Vervolgens werd een systeem ontwikkeld waarin het verloop van

binding en metabolisme van 1,25-$(OH)_2D_3$ in intacte OB (sarcoom) cellen (UMR-106) kon worden bestudeerd. Het verloop van de specifieke binding van 1,25-$(OH)_2D_3$ in UMR-106 cellen werd gekarakteriseerd door (a) een initieel snelle toename van de binding, die de associatie van het hormoon met de receptor representeert, (b) een maximale binding na 90-120 min en vervolgens (c) een snelle afname van de binding, die nauw gecorreleerd is met de afname van de 1,25-$(OH)_2D_3$ concentratie door zijketen-oxidatie van het hormoon. Remming van het metabolisme met behulp van ketoconazol resulteerde na een interval van 90 min in een sterke toename van de hoeveelheid specifiek gebonden 1,25-$(OH)_2D_3$. Deze zogenaamde '1,25-$(OH)_2D_3$ receptor up-regulation' lijkt ligand-afhankelijk te zijn. Zij is gesuperponeerd op een 'up-regulation' veroorzaakt door een nog onbekende factor gerelateerd aan de medium-verversing bij de start van het experiment. Dat actinomycine D de toename van binding tegengaat wijst op de betrokkenheid de novo mRNA-synthese bij de 'up-regulation' van de 1,25-$(OH)_2D_3$ receptor. Anderzijds werden ook enige aanwijzingen verkregen dat binding van 1,25-$(OH)_2D_3$ leidt tot een minder snelle afbraak van de receptor. Dientengevolge is het waarschijnlijk dat de ligand-afhankelijke 'up-regulation' van de 1,25-$(OH)_2D_3$ receptor wordt bepaald door zowel een toename van de synthese van de receptor als ook door een toename van de half-waarde tijd van het receptor-eiwit. Bovendien werd aangetoond dat het door 1,25-$(OH)_2D_3$ geïnduceerde eigen metabolisme een tweeledig effect heeft: (1) *direct* doordat het de intracellulaire concentraties van 1,25-$(OH)_2D_3$ kan reguleren en (2) *indirect* door modulatie van de homologe regulatie van de 1,25-$(OH)_2D_3$ receptor.

Bovengenoemde resultaten wijzen er op dat de homologe 'up-regulation' van de receptor een mogelijkheid geeft, door middel van het versterken van het primaire hormonale signaal, de gevoeligheid van het doelwit-orgaan voor relatief kleine schommelingen in de 1,25-$(OH)_2D_3$ spiegels te verhogen. Anderzijds biedt de snellere afbraak van 1,25-$(OH)_2D_3$ bij een hogere receptor-dichtheid de mogelijkheid een opwaartse spiraal van receptor-'up-regulation' te doorbreken.

In hoofdstuk 5 worden de resultaten beschreven van een relatief kortdurende behandeling (3 maanden) met 1α-$(OH)D_3$ op de botombouw activiteit en de mineralisatie bij predialyse patiënten met een renale osteodystrofie. Negenentwintig patiënten met een kreatine klaring van 17.6 ± 1.7 ml/min werden behandeld met 1α-$(OH)D_3$ (gemiddelde dosering 0.55 ug/dag). Bij het begin van het onderzoek hadden 14 patiënten een osteoblastzoom-lengte van minder dan 4% (groep I) en hoge osteoïd parameters, terwijl bij 13 patiënten met een osteoblastzoom-lengte van meer dan 4% zowel hogere iPTH-spiegels als een histologisch beeld passend bij hyperparathyreoïdie werd gevonden. Behandeling met 1α-$(OH)D_3$ resulteerde in een aanzienlijke suppressie van de secundaire hyperparathyreoïdie in groep II met een significante afname van zowel de osteoblastzoom-lengte als het percentage trabeculair bot bezet met een dubbele tetracycline merking. De vóór de behandeling

reeds normale mineraal-appositie-snelheid veranderde door behandeling niet. Dit wijst er op dat de afname van het percentage tetracycline dubbel label een afname van het aantal 'Basic Multicellular Units' representeert en niet een verlaagde bot-appositie-snelheid. In groep II resulteerde behandeling met 1α-$(OH)D_3$ in een positieve correlatie tussen osteoblastzomen en het aantal osteoclasten, hetgeen een rol suggereert voor $1,25\text{-}(OH)_2D_3$ in een koppeling tussen osteoblasten en osteoclasten. Tenslotte bestond er een negatieve correlatie (berekend voor beide groepen tezamen) tussen het serum calcium en de osteoïedzoom-lengte, terwijl het calciumgehalte van het serum positief was gecorreleerd met het met thionine gekleurde mineralisatie-front, indien uitgedrukt als percentage van de osteoïedzoom-lengte, maar niet indien berekend als percentage van de osteoblastzomen. Dit gegeven steunt de hypothese dat de toename van het mineralisatie-front na 3 maanden behandeling niet tot stand komt via een direct effect van $1,25\text{-}(OH)_2D_3$ op de osteoblast, maar indirect via de door $1,25\text{-}(OH)_2D_3$ geïnduceerde toename van het calciumgehalte van het serum.

Nawoord

Het uitvoeren van een promotie-onderzoek is een taak die men zeker niet geheel zelfstandig tot een goed einde kan brengen. Deze stelling wint nog aan kracht indien een clinicus zich gaat bezighouden met een onderzoeksgebied waarvoor verschillende cel-biologische en biochemische vaardigheden noodzakelijk zijn. Daarom wil ik op deze plaats diegenen bedanken die mij gestimuleerd en geholpen hebben bij het voltooien van dit proefschrift.

Allereerst Jacques Lockefeer die naast de opleiding tot internist de mogelijkheid schiep een begin te maken met het onderzoek dat uiteindelijk tot dit boekje heeft geleid.

Het is mijn eerste promotor, Jan Birkenhäger, die vanaf het allereerste begin de koers heeft bepaald en mij van voldoende intellectuele brandstof voorzag om ook op moeilijke momenten door te zetten. Met veel genoegen denk ik terug aan de beginperiode, waar Jan Birkenhäger 's ochtends om 7 uur reeds paraat was om te helpen bij het uitpreparerèn van kippe-calvaria. Deze geweldige inzet tezamen met zijn ideeën voor verder onderzoek waren en blijven van onschatbare waarde.

Ook mijn tweede promotor, Theo Visser, heeft vanaf het begin op geduldige en vriendelijke wijze de vele en soms verwarrende hypothesen aangehoord. Dat één en ander uiteindelijk toch tot een promotie-onderzoek heeft geleid is mede aan zijn heldere analyse van de resultaten te danken.

De overige leden van de promotiecommissie, Prof. Dr. J.F. Jongkind en Dr. P.J. Nijweide ben ik erkentelijk voor hun bereidheid het manuscript zo snel te beoordelen.

Bij het uitvoeren van de vele experimenten was de hulp van Hans Peter Schilte onontbeerlijk. Zijn grote inzet en betrokkenheid bij het onderzoek zijn mij zeer tot steun geweest. Ook Cok Buurman ben ik dank verschuldigd voor alle hulp bij de eerste wankele stappen in het laboratorium.

Naast de hulp van de medewerkers van het laboratorium in Dijkzigt, dient ook de hulp vermeld te worden van Han Scherft, Nel Herrmann-Erlee en Peter Nijweide (Laboratorium voor Celbiologie en Histologie, Leiden) bij het opzetten van de verschillende osteoblast-kweken.

Dorie Birkenhäger ontsluierde met veel geduld de geheimen van de histomorfometrie. Samen met o.a. Jeltje Zeelenberg beoordeelde zij op deskundige wijze de botbiopten.

De statistische analyse van de gegevens voortkomend uit het patiëntenonderzoek

zijn verricht door Frank van Berkum. De bijdrage die hij hiermee heeft geleverd kan als uiterst significant worden gekenmerkt.

De medewerkers van de Balans-afdeling, met name Joke van Vuure, Cootje van Aller en Marijke Hengeveld, bedank ik voor de vele kopjes koffie en de plezierige sfeer tijdens de jarenlange samenwerking. Ook de vriendelijke wijze waarop zij tijdens het nemen van de botbiopten de patiënten op hun gemak stelden is onnavolgbaar.

Dieneke van Wessem verzorgde de literatuurlijst. Zij was het ook die met grote blijmoedigheid hulp verleende toen op het laatste moment een deel van een der hoofdstukken door de tekstverwerker werd uitgewist.

Roel Docter fungeerde diverse malen als 'trouble-shooter' die problemen met de verschillende biochemische technieken oploste. Ook zijn kennis van de tekstverwerker heeft mij uit angstige situaties gered.

Ik dank Jos van Geelen (Afdeling Inwendige Geneeskunde I, Dijkzigt Ziekenhuis), Piet Kramer (Afdeling Nefrologie, Clara Ziekenhuis) Piet Hein Hillen, Pim van Boven en Job Juttmann (Afdeling Inwendige Geneeskunde, St Elisabeth Ziekenhuis, Tilburg) voor de hulp bij het patiëntenonderzoek, waarvan slechts een deel in dit proefschrift wordt behandeld. Ook de patiënten wil ik mijn oprechte dank betuigen voor hun loyale medewerking aan het onderzoek.

De Audiovisuele Dienst komt alle eer toe voor het op uitstekende wijze tekenen van de gepresenteerde figuren.

Leo Pharmaceuticals maakte het drukken van dit proefschrift mogelijk. Aan de contacten met Henk Reiling bewaar ik de beste herinneringen.

Curriculum vitae

De schrijver van dit proefschrift werd geboren op 29 juli 1952 te Dordrecht. In 1971 werd het diploma HBS-B behaald aan het Gemeentelijk Lyceum te Dordrecht. Aansluitend studeerde hij Geneeskunde aan de Medische Faculteit van de Erasmus Universiteit, alwaar op 16 december 1977 het artsexamen werd afgelegd. Van 1978 tot en met 1983 was hij arts-assistent op de afdeling Inwendige Geneeskunde van het St Elisabeth Ziekenhuis te Tilburg (hoofd Dr. V.A.M. Terwindt; vervolgens Dr. J.H.M. Lockefeer). Eind 1981 werd gedurende 4 maanden onderzoek verricht op het laboratorium voor Celbiologie en Histologie van de Rijksuniversiteit Leiden (hoofd Prof. Dr. J.P. Scherft). Op 1 februari 1983 volgde inschrijving in het specialistenregister, waarna als wetenschappelijk medewerker van de afdeling Inwendige Geneeskunde III (hoofd Prof. dr. J.C. Birkenhäger) het in dit proefschrift beschreven onderzoek werd verricht. Vanaf 1 september 1986 is hij als internist aan voornoemde afdeling verbonden.