REGULATION OF 1,25-DIHYDROXYVITAMIN D_3 RECEPTORS IN HUMAN PROMYELOCYTIC LEUKEMIC CELLS, HL-60

by

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REGULATION OF 1,25-DIHYDROXYVITAMIN D_3 RECEPTORS IN HUMAN PROMYELOCYTIC LEUKEMIC CELLS, HL-60

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The regulation of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) was investigated in human target cells using human promyelocytic leukemic cell line, HL-60, as a model system. HL-60 cells were cultured successfully in serum-free chemically defined medium (SFM) in order to remove undesirable factors present in serum. Cells grown in SFM retained a full capacity to differentiate to monocytic pathway in response to 1,25-$(OH)_2D_3$.

Receptors for 1,25-$(OH)_2D_3$ in intact HL-60 cells cultured in SFM were saturated with [^{3}H]1,25-$(OH)_2D_3$ within 1 h after hormone addition (basal level). Continuous exposure of cells to hormone for longer times resulted in an apparent up-regulation (an increase in specifically bound [^{3}H]1,25-$(OH)_2D_3$) which was maximal between 8-16 h after hormone addition. This was followed by an apparent down-regulation (a loss of specifically bound [^{3}H]1,25-$(OH)_2D_3$) which could be observed by 24 h. The physical/chemical characteristics of receptors were not changed significantly in up- and down-regulated receptors as indicated by Scatchard analysis, sucrose gradient density analysis, DNA-cellulose chromatography and fast protein liquid chromatography. However, the estimated number of receptor sites was increased 2-fold by 14 h followed by a decrease in the number of receptor sites to the basal receptor level by 24 h.

The immunoblotting assays indicated that an up-regulation resulted from actual increases in receptor protein and a down-regulation from a decrease in

receptor protein and a loss of binding ability of receptors. The up-regulation of 1,25-$(OH)_2D_3$ receptors required new mRNA and protein synthesis, while down-regulation did not appear to require new protein synthesis.

Both The monocytic inducers of HL-60, 1,25-$(OH)_2D_3$ and phorbol 12-myristate 13-acetate (PMA) could regulate the level of receptors. However, the granulocytic inducers of HL-60 cells, retinoic acid and dimethyl sulfoxide failed to regulate the specific [^{3}H]1,25-$(OH)_2D_3$ binding. The present study suggests that the regulation of 1,25-$(OH)_2D_3$ receptors in HL-60 cells might be one of the earliest phenotypic changes which might be associated with the monocytic differentiation process.

Key Words: 1,25-dihydroxyvitamin D_3 receptor; human promyelocytic leukemic cell line; immunoblotting assay.

William D Mell

DEDICATION

This thesis is dedicated to my husband, Kyungmann Kim, and my parents, Bokhee and Sungho Lee. I am very grateful for their ever present love, support and encouragement.

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CHAPTER I

INTRODUCTION

GENERAL

Vitamin D is essential for life in higher animals. It is one of the most important biological regulators of calcium metabolism. Along with the two peptide hormones calcitonin and parathyroid hormone, vitamin D is responsible for the minute-to-minute as well as the day-to-day maintenance of calcium homeostasis. These three substances also play an important role in phosphorous homeostasis. In the absence of vitamin D, a host of problems arise, ranging from those that affect the whole organism (e. g., inhibition of growth), to those that affect particular cell types (e. g., changes in the morphology of the columnar epithelial and/or goblet cells of the intestinal mucosa), and ultimately to those that are manifest at the subcellular level (e. g., alteration of the morphology of kidney mitochondria). In view of the obvious dependence of normal life in higher animals on the continued presence of vitamin D, it is not surprising that scientists have exerted a major effort to understand the physiological, cellular, subcellular, and molecular mode of action of this vital substance. Certainly vitamin D is unique among the vitamins inasmuch as it is the only vitamin known to be a precursor of a hormone and its action is truly hormonal in nature.

HISTORICAL BACKGROUND

Man is reported to have been aware in a general sense of vitamin D since early antiquity (Solecki 1971 a, b). Vitamin D was discovered as a consequence of rickets, a disease which had become a serious problem in the Western world (DeLuca, 1978). Rickets and osteomalacia are the juvenile and adult forms of a single group of disorders characterized by deficient mineralization of bone and

cartilage. The first scientific description of rickets was provided by both Dr. Daniel Whistler (1645) and Professor Francis Glisson (1650 a, b) in the seventeenth century. The two most important proposals concerned the relationship between lack of sunlight and incidence of rickets and the possibility that rickets was induced by a lack or deficiency of a nutritional factor. However, at this time, the close interdependency of these two relationships was not at all appreciated.

The major breakthrough in understanding the causative factors of rickets was the development of nutrition as an experimental science and the appreciation of the existence of vitamins. In this respect the leadership roles of Sir F. Gowland Hopkins (1920) and Professor C. Funk (1914) should be acknowledged. Furthermore, Sir Edward Mellanby unequivocally established that rickets was caused by a deficiency of a trace component present in the diet (Mellanby 1919, 1921). He established that cod-liver oil was an excellent antirachitic agent and attributed the antirachitic activity to the newly described vitamin A (McCollum and Davis, 1913), but McCollum et al. (1922) later demonstrated experimentally the distinctive properties of vitamin A and the antirachitic factor. In their historic paper, they demonstrated that the antirachitic activity of cod-liver oil could survive both aeration and heating, whereas the antixerophthalmic factor, or vitamin A, was inactivated by this process. McCollum first called the antirachitic factor "the forth vitamin" and later named the substance 'vitamin D' (McCollum et al., 1925).

However, the most significant advance was an appreciation of the importance of ultraviolet light or radiant energy to the cure and etiology of rickets. Huldschinsky (1919, 1919-1920) first showed that the uv rays from a mercury-vapor lamp was quite effective in increasing the degree of calcification of the epiphysis of rachitic infants and these results were quickly reproduced in

experimental animals by Hess (1922) and Hess and Gutman (1922). However, no connection was made between the mysterious curative powers of ultraviolet light and cod liver oil until the work of Goldbatt and Soames (1923) and Steenbock and Black (1924). Both groups found that irradiated livers from rachitic rats and food had acquired the property of being "antirachitic". Hess and Weinstock (1925 a, b, c) confirmed the dictum that "light equals vitamin D". They excised a small portion of skin, irradiated it with uv light, and then fed it to groups of rachitic rats. This diet provided an absolute protection against rickets whereas the unirradiated skin provided no protection.

It was soon proven by Steenbock and Black (1925) and Hess and Weinstock (1925 a, b, c) that the antirachitic factor was confined to the nonsaponifiable fraction of the fats, particularly sterols. The precursor of vitamin D (provitamin) was identified as 'ergosterol or highly unsaturated sterol of similar constitution which is converted to vitamin D by irradiation' (Rosenheim and Webster, 1927). Today, we know ergosterol to be the precursor of vitamin D_2, a physiologically active analog of vitamin D_3. In 1932, Windaus et al. and Askew et al. simultaneously identified the structure of vitamin D_2 from irradiated plant sterol as a secosterol and Windaus et al. (1936) determined the structure of the antirachitic factor, vitamin D_3 that resulted after uv irradiation of synthetic 7-dehydrocholesterol. A rich source of 7-dehydrocholesterol was found in pig skin (Windaus and Bock, 1937). Thus it was concluded that vitamin D_3 was the natural antirachitic substance in animals and was produced when 7-dehydrocholesterol in the skin was irradiated with the ultraviolet component of sunlight.

BIOGENESIS AND METABOLISM OF VITAMIN D_3

The skin is in possession of a potent sterol-biosynthesizing system (DeLuca et al., 1971). This system produces large amounts of 7-dehydrocholesterol, which are readily found in the epidermis (Idler and Baumann, 1952) and ultraviolet light of approximately 300 nm wavelength readily penetrates skin to the level of epidermis (Daniels, 1964). Vitamin D_3 is produced in the skin from 7-dehydrocholesterol by an ultraviolet light induced photolysis followed by thermally dependent isomerization (Esvelt et al., 1978) (Fig. 1).

The modern era of the study of vitamin D became possible because of the availability of radioactive vitamin D with high specific activity. Kodicek and co-workers (Kodicek, 1956) were the first to demonstrate the metabolism of vitamin D into more polar substances devoid of biological activity. The first detailed study of the metabolism of physiological doses of vitamin D was carried out by Norman et al. (1964) and Lund and DeLuca (1966). The methanol-chloroform extraction of tissues followed by gradient elution chromatography indicated at least four metabolites of vitamin D, the major metabolite of which was more active than the parent vitamin D. This was the first clear-cut demonstration of the existence of a biologically active metabolite of vitamin D.

The product of the photolysis reaction is previtamin D_3, which slowly equilibrates in the skin to vitamin D_3. The formed vitamin D_3 is then bound to the plasma transport protein to be transported to the liver where vitamin D_3 undergoes its first metabolic activation step in which it is converted to 25-hydroxyvitamin D_3 (25-OH-D_3) by a microsomal P_{450} hydroxylase in a reaction that involves NADPH and molecular oxygen (Blunt et al., 1968; Madhok et al., 1978) (Fig. 2). This compound is the major circulating form of vitamin D being at a level of 20-30

ng/ml. Although extrahepatic 25-hydroxylases have been reported (Tucker et al., 1973; Bhattacharyya and DeLuca, 1974), the physiological importance remains relatively unknown. 25-OH-D_3 is transferred to the kidney via a transport protein where it undergoes its next activation reaction to form 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) in mitochondria of the proximal convoluted tubule cells (Haussler et al., 1968; Brunette et al., 1978; Ghazarian et al., 1974).

25-OH-D_3 undergoes a variety of other metabolic conversions. In the kidney, intestine and cartilage there exists 25-OH-D_3 24R-hydroxylase which converts 25-OH-D_3 to 24α,25-dihydroxyvitamin D_3 (24R,25-$(OH)_2D_3$) (Holick et al., 1972; Knutson and DeLuca, 1974). This compound has proven to be a major metabolite of vitamin D_3 whose exact funcion is still under examination. There is a stimulation and accumulation of 1,25-$(OH)_2D_3$ and a suppression of 24,25-$(OH)_2D_3$ when hypocalcemia appears. On the other hand, when calcium is no longer needed, the production of 1,25-$(OH)_2D_3$ is suppressed and, instead, 24-hydroxylation occurs. This enzyme also produces 1,24R,25-trihydroxyvitamin D_3 (1,24R,25-$(OH)_3D_3$) from 1,25-$(OH)_2D_3$ *in vivo* (Holick et al., 1973; Kleiner-Bossaller and DeLuca, 1974). This compound was about one-half as active as 1,25-$(OH)_2D_3$ in the rat with respect to mineralization or mobilization of calcium from bone and stimulation of intestinal calcium transport (Boyle et al., 1973). 25-OH-D_3 is converted to 25R,26-$(OH)_2D_3$ in the kidney and elsewhere (Boris et al., 1977) which is weakly active in stimulating intestinal calcium transport and has little activity in the other systems known to be responsive to vitamin D_3. The physiological function of these metabolites remains unknown at the present time.

1,25-$(OH)_2D_3$ undergoes a very rapid side chain cleavage reaction giving rise to 24,25,26,27-tetranor, 1α-OH-D_3 23-carboxylic acid or calcitroic acid in

liver and possibly intestine (Esvelt et al., 1979). Its biological activity is minimal and it is unknown whether it plays a physiological role or whether it represents a major inactivation mechanism. It is apparent that an active catabolic mechanism exists to inactivate these potentially toxic secosteroids. 1,25-$(OH)_2D_3$ has a life time about 5 hours in human plasma. Vitamin D compounds are primarily excreted into the bile, resulting ultimately in excretion in the feces. Since 1,25-$(OH)_2D_3$ is made exclusively in the kidney and has its function in intestine, bone and elsewhere, it is considered a hormonal substance.

PHYSIOLOGICAL FUNCTION OF VITAMIN D

The major function of vitamin D and hence of 1,25-$(OH)_2D_3$, the most biologically active form of vitamin D, is to elevate plasma calcium and phosphorous concentration to super saturation levels. Supersaturating levels of calcium and phosphorous are required to support normal mineralization of newly forming bone. Elevated calcium levels of blood are also necessary for normal function of nerve and muscle and prevent such diseases as hypocalcemic tetany and muscle weakness. Calcium and phosphorous are the most abundant of the inorganic elements in the body (Widdowson and Dickerson, 1964). The system of calcium and phosphorous homeostasis involves the integrated actions of the site of uptake, the intestine, with those of the major site of deposition of these ions, the bone, and of the major site of excretion of these minerals, the kidney.

A. Intestinal Effect of Vitamin D

Intestinal calcium absorption is an active transport process. It has been adequately demonstrated that calcium is transported from the lumen of intestine to

the serosal fluid by a process which requires metabolic energy (Martin and DeLuca, 1969; Wasserman et al., 1961; Schachter, 1963). The transfer occurs against an electrical and concentration gradient. 1,25-$(OH)_2D_3$ brings about an elevation of plasma calcium concentration by stimulating the active transport of calcium in the small intestine from lumen to blood (DeLuca, 1978; Nicolaysen, 1937). Besides metabolic energy the only other known requirement is sodium ions. This requirement for sodium is believed to be necessary for the expulsion of calcium across the basal-lateral membrane, and in its absence calcium accumulates in the intestinal villus cells (Martin and DeLuca, 1969). Phosphate is the normal accompanying anion, although this transport mechanism does not require the presence of phosphate to operate (Martin and DeLuca, 1969). Most investigators agree that vitamin D must function at the mucosal surface membrane.

It has been visualized that 1,25-$(OH)_2D_3$ must function in a manner similar to that of the steroid hormone (Chen and DeLuca, 1973; Brumbaugh and Haussler, 1974, 1975). There is a good evidence that 1,25-$(OH)_2D_3$ binds to a intracellular receptor that then interacts with chromatin to induce synthesis of proteins which play a role in both metabolism of 1,25-$(OH)_2D_3$ and transport of calcium across the intestine (Stohs et al., 1967; Hallick and DeLuca. 1969; Zerwekh et al., 1976). There is considerable question as to what is the nature of the calcium transport proteins or substances which are induced by 1,25-$(OH)_2D_3$. Among such proteins, the calcium binding protein (CaBP) was described originally in the chick, which appeared only after administration of vitamin D (Wasserman and Taylor, 1968). However, it has been reported that the stimulation of calcium transport preceded the induction of CaBP protein (Spencer et al., 1976) and the decay of calcium transport occurs more quickly than the disappearance of CaBP (Haussler and McCain,

1977), leading to the conclusion that this protein may not be involved in the initiation of the calcium transport process but the protection of the cell from the increased intracellular calcium concentration (Bikle et al., 1983). Recently, Bishop et al. (1983, 1984) reported that CaBP is induced within 1-2 h exposure to 1,25-$(OH)_2D_3$ using the extremely sensitive technique and precedes the onset of the calcium uptake response by at least 2 h. These data together with the finding that the translatable mRNA for CaBP appeared within 2 h after dosing with the 1,25-$(OH)_2D_3$ (Spencer et al., 1976) suggest that CaBP may be involved in the initiation of the calcium transport process.

It is known that maximal vitamin D stimulation of calcium uptake occurs in the duodenum, whereas maximal stimulation of phosphate occurs in the jejunum (Brumbaugh and Haussler, 1975; Koworski and Schacter, 1969; Harrison and Harrison, 1961). In the jejunum phosphate transfer in an active process (Walling, 1977) and requires the presence of sodium ions (Taylor, 1974). Furthermore, this process does not require the presence of calcium in the ambient fluid, which implicate this mechanism is independent of the calcium transport mechanism responsive to 1,25-$(OH)_2D_3$. Little is known concerning the mechanism of phosphate transfer and so far a specific phosphate binding protein has not yet been demonstrated. Likely much new work can be expected in regard to the mechanism of action of 1,25-$(OH)_2D_3$ at its target sites.

B. Effect on Bone Mineral Metabolism

Bone Accretion. The mobilization of calcium from bone has been much more difficult to study for technical reasons, although it is possible by means of tissue culture experiments to study the process *in vitro* (Trummel et al., 1969; Raisz

et al., 1972; Reynolds et al., 1973). Nearly 99% of calcium and phosphorous ions reside in the hydroxyapatite crystals of bone, with the remainder being present in extracellular fluids and other cellular compartments. Thus, bone tissue provides an enormous reservoir of both calcium and phosphorous. Mineralization of bone is unquestionably impaired in the absence of vitamin D giving rise to the classical nutritional deficiency known as rickets. Lack of calcification in rickets may perhaps be due to reduced plasma Ca levels as a consequence of reduced bone mobilization and reduced intestinal absorption together with increased renal excretion (DeLuca, 1967). On the other hand, it has been considered reasonable that the uptake of mineral ions by bone and their utilization during calcification may be controlled directly by a metabolite of vitamin D. Migicovsky (1957) reported that vitamin D increase the rate of calcium accretion into bone after administering either oral or intramuscular doses of $^{45}Ca^{++}$ (to obviate effects of vitamin D on intestinal calcium absorption). Eastwood et al. (1974) found that in patients with chronic renal failure the associated osteomalacia did not respond to large intakes of calcium sufficient to maintain near normal plasma levels. However, evidence for a direct and vital role for vitamin D in bone formation remains inconclusive as yet.

Bone Resorption. Resorption of bone, involving the dissolution of both bone mineral and matrix, is a normal part of bone growth and maintenance. Vitamin D can only cause resorption *in vivo*, being completely inert if added to the bone culture system. Although a number of the analogues of vitamin D can stimulate bone resorption *in vitro* the information presently available suggests that 1,25-$(OH)_2D_3$ is the active form of vitamin D_3 regulating the rate at which this process occurs *in vivo*. In addition, 1,25-$(OH)_2D_3$ is the most potent substance known with calcium mobilizing activity whether measured *in vivo* (Wong et al.,

1972) or *in vitro* (Reynolds, 1974; Stern et al., 1975). The mechanism by which this steroid stimulates bone resorption remains unknown. The studies with actinomycin D (Einstein and Passavoy, 1964) imply that the effect requires continuing protein synthesis and by analogy with the intestinal events suggests that the synthesis of proteins involved in resorption is controlled at the transcriptional stage by 1,25-$(OH)_2D_3$. The specific nuclear localization of 1,25-$(OH)_2D_3$ in the target cells was detected in osteoblasts and bone lining cells but not in osteoclasts (Stump et al., 1981; Narbaitz, 1983). A stable cell line and primary culture of osteoblasts contains also the receptors for 1,25-$(OH)_2D_3$ and the magnitude of hormonal responsiveness, such as inhibition of collagen synthesis or 24-hydroxylase activity, is directly correlated with the abundance of receptor, which implicated the important role of 1,25-$(OH)_2D_3$ in osteoblast functions (Walters et al., 1982: Chen and Feldman, 1985).

Parathyroid hormone stimulates adenyl cyclase activity leading to an accumulation of cyclic AMP in bone cells before mobilization of bone mineral occurs (Chase and Aurbach, 1970; Wells and Lloyd, 1969; Rasmussen et al., 1968). This hormone increases RNA and protein synthesis in osteoclasts, and puromycin and actinomycin D inhibit the calcium mobilizing response to parathyroid hormone (Rasmussen et al., 1964; Tashjian et al., 1964, Kunin and Krane, 1965). Since the hormone had no effect on calcium mobilization from bones of vitamin D-deficient animals, the administration of parathyroid hormone to rachitic animals may increase cAMP levels but not calcium mobilization if some later step was rate limiting. The 1,25-$(OH)_2D_3$ also increase phosphate mobilization from bone of parathyroidectomized rats (Castillo et al., 1975). The

parathyroid hormone-independent bone mineralizing action of 1,25-$(OH)_2D_3$ mainly affects bone phosphate but some mobilization of calcium also occurs.

C. Vitamin D Actions in Kidney

Only 0.5-1.0% of filtered calcium in kidney is lost in urine, which is a reflection of the remarkably effective tubular reabsorption mechanisms for calcium. Taylor and Wasserman (1972) first reported the presence of a vitamin D-dependent calcium-binding protein (CaBP) in the kidney of chicks and the dependence of this renal CaBP on the presence of vitamin D. However, as in the case for the intestinal CaBP, no conclusive studies have yet been presented showing a functional involvement of the renal CaBP in the renal metabolism of calcium. There is some evidence that 1,25-$(OH)_2D_3$ stimulates renal reabsorption of calcium in the distal tubules (Steele et al., 1975), but the effect of vitamin D on renal calcium excretion in man is not yet clearly understood. Hall et al. (1969) and Brickman et al. (1974) reported an increase in urinary calcium after administration of large doses of vitamin D_3 or 1,25-$(OH)_2D_3$. A possible explanation for these observations is that the vitamin D or 1,25-$(OH)_2D_3$ mediated an increased tubular reabsorption of calcium and a slight elevation of serum calcium would result, which would present a higher filtered load of calcium to the kidney tubule; the response, then would more likely be a decrease in the fractional tubular reabsorption of calcium and modest hypercalciuria.

80-90% of inorganic phosphate filtered at the glomerulus is reabsorbed by renal tubule. A significant increase in phosphate reabsorption following the infusion of vitamin D in parathyroidectomized and intact rats has been reported using a sophisticated micropuncture technique (Gekle et al., 1971). However, the

precise relationship between the effects of 1,25-$(OH)_2D_3$ and PTH, whether they are permissive or independent, has not yet been determined. The detailed responsibility of 1,25-$(OH)_2D_3$ in maintaining homeostasis of phosphate at the kidney level remains to be clearly established.

RECEPTORS FOR 1,25-$(OH)_2D_3$

With the identification of 1,25-$(OH)_2D_3$ as a hormone derived from vitamin D, it was postulated that 1,25-$(OH)_2D_3$ functions in a manner similar to that of other steroid hormones (DeLuca, 1974). While the metabolism of vitamin D to 1,25-$(OH)_2D_3$ was being elucidated, parallel investigations with other steroid hormones were establishing the importance of receptors in the mechanism of hormone action. Glasscock and Hoekstra (1959) and Jensen and Jacobson (1960) reported in their studies on the fate of estrogens in target tissues that the initial interaction of steroid hormones with target tissues became an active area of research (Gorski et al., 1968). Glasscock and Hoekstra (1959) reported that when synthetic estrogen, labeled with high specific activity using tritium, was administered to young female goats and sheep, target tissues showed higher uptake and prolonged retention of the hormone. Since most of the radioactive estrogen in the uterus was associated with the 'nuclear-myofibrillar fraction' and the binding sites for estrogen were stereospecific for estrogen molecule and the estrogen was released by protease, it was hypothesized that the estrogen was bound to a protein (Noteboom and Gorski, 1965). The first direct demonstration of the estrogen receptor was accomplished using sucrose density gradients (Toft and Gorski, 1966). The hypothesis that estrogen regulates gene expression (Mueller et al., 1958) was

supported by experiments demonstrating that estrogen stimulates protein and RNA synthesis (Muller et al., 1960; Noteboom and Gorski, 1963; Gorski et al., 1968).

As early as 1965 attention was focused on the action of vitamin D and its metabolites in the nuclei of intestinal mucosal cells. In addition, specific nuclear localization of 1,25-$(OH)_2D_3$ was demonstrated in osteoblasts and lining cells of bone (Stumpf et al., 1981; Narbaitz et al., 1983), distal renal tubular cells of kidney (Stumpf et al., 1980; Narbaitz et al., 1982), the malpighian layer of skin, the pituitary (Stumpf et al., 1981), islet cells of the pancreas (Clark et al., 1980), certain cells of brain (Stumpf et al., 1982), and mammary gland (Narbaitz et al., 1981). Therefore, in addition to the known target organs of 1,25-$(OH)_2D_3$ (intestine, kidney, bone), specific nuclear localization was demonstrated in tissues not yet known to be target organs. There is no doubt that nuclear localization of 1,25-$(OH)_2D_3$ in target cells precedes onset of organ response. This strongly suggests that the mechanism of action of 1,25-$(OH)_2D_3$ in known target organs is nuclear mediated (DeLuca et al., 1982).

The first report of a specific receptor-like protein for 1,25-$(OH)_2D_3$ appeared in 1973 (Brumbaugh and Haussler, 1973 a, b) but firmly was established in 1976 (Kream et al., 1976). Since that time, many studies have been carried out demonstrating the existence of a macromolecule in a variety of tissues that specifically binds 1,25-$(OH)_2D_3$ with high affinity and low capacity (Franceschi et al., 1981) including the small intestine of the chicken, rat intestine (Kream and DeLuca, 1977; Feldman et al., 1979), rat embryonic bone (Kream et al., 1977) and rachitic chick bone (Mellon and DeLuca, 1980). Thorough studies of the physical parameters of the receptor molecule (Mellon and DeLuca, 1979; Wecksler and Norman, 1980) revealed that the avian species have a receptor sedimenting at 3.7S,

whereas the mammalian species have a receptor sedimenting at 3.2S. The eqilibrium dissociation constant (Kd) for the receptor molecule ranges between 10^{-10} and 5×10^{-11} and molecular weight ranges between 50,000-70,000 daltons. The salient features of this receptor are that it is a DNA-binding protein with high affinity and specific binding of 1,25-$(OH)_2D_3$ (Pike and Haussler, 1979). Its hormone-binding and DNA-binding domains are sensitive to sulfhydryl-blocking reagents. Proteolysis of 60,000 dalton chick intestinal receptors with a low concentration of trypsin results in the production of a 40,000-45,000 MW fragment that binds hormone but no longer binds DNA (Allegretto and Pike, 1985). Concomitantly, a 20,000 MW fragment that does not bind hormone but associates with DNA with native affinity appears. The current model for the chick intestinal receptor visualizes it as a rod-shaped molecule with at least two distinct domains, one for binding the 1,25-$(OH)_2D_3$ ligand and a second that corresponds to the 20,000 MW fragment and contains the DNA-binding site.

MONOCLONAL ANTIBODIES TO THE 1,25-$(OH)_2D_3$ RECEPTOR

A new era of steroid hormone receptor research was begun in 1977 when Jensen and co-workers reported the production of antibody to the estrogen receptor (Greene et al., 1977). Antibodies provided for the first time a way to recognize receptor proteins independently of steroid binding. It has been demonstrated that estrogen receptors from different species are immunologically similar and that estrogen receptors are immunologically different from other steroid hormone receptors, such as those for progesterone and testosterone (Greene et al., 1977).

Although the specificity of the antisera for the estrogen receptor made them alternative probes for examining the structure and function of estrogen receptors,

such studies were limited by the heterogeneity of the antibody preparation (Greene et al., 1981). The production of antisera also requires the use of pure antigen, which is very difficult, if not impossible, to obtain. In 1975 Kohler and Milstein fused mouse myeloma cells with spleen for growth of the resulting hybridomas. The hybrids they produced secreted antibodies directed against the antigen originally used to immunize the mice. This technology is particularly well suited to the production of antibodies to impure proteins since it is possible to select hybridomas that secrete antibodies to the specific protein of interest, provided the proper assays are available. Since then, monoclonal antibodies have been produced to the progesterone receptor (Logeat et al., 1983; Radanyi et al., 1983) and the glucocorticoid receptor (Westphal et al., 1982; Grandics et al., 1982; Okret et al., 1984). Monoclonal antibodies to a number of cell surface receptors, including those for thyrotropin (Yavin et al., 1981), insulin (Roth et al., 1982; Kull et al., 1982), asialoglycoproteins (Hartford and Ashwell, 1981), epidermal growth factor (Schreiber et al., 1981), acetylcholine (Tzartos and Lindstrom, 1980) and transferrin (Trowbridge and Omary, 1981) have also been developed.

Monoclonal antibody technology has recently been applied to the study of the 1,25-$(OH)_2D_3$ receptor. Hybrids producing antibodies to the 1,25-$(OH)_2D_3$ receptor from chicken intestine were detected by double antibody immunoprecipitation (Pike et al., 1982). These monoclonal antibodies have been used to aid studies of 1,25-$(OH)_2D_3$ receptor structure by proteolysis (Allegretto and Pike, 1985). They have been also used to demonstrate that deficiencies in hormone binding associated with the disease vitamin D-dependent rickets type II probably arise from structural variations in the receptor molecule, not lack of expression of the protein (Pike et al., 1984). Monoclonal antibodies to the porcine

intestinal 1,25-$(OH)_2D_3$ receptor have been produced (Dame et al., 1986) and employed for the characterization of porcine 1,25-$(OH)_2D_3$ receptor as a 55 KDa protein with the major form having an isoelectric point of 6.1 (Dame et al., 1985; Pierce et al., 1987).

1,25-$(OH)_2D_3$ RECEPTORS IN THE HEMATOPOIETIC SYSTEM

Sites for the 1,25-$(OH)_2D_3$ receptor include cells derived from bone marrow and thymus, suggesting that 1,25-$(OH)_2D_3$, like the glucocorticoids and other steroids, may be an immunoregulator. There are several reports that monoblastic and myeloblastic cell lines are differentiated into macrophages in the presence of physiological levels of 1,25-$(OH)_2D_3$ (Abe et al., 1981; Mangelsdorf et al., 1984; Olsson et al., 1983). Two of these human cell lines, HL-60 and U-937, possess classic 1,25-$(OH)_2D_3$ receptors. The resting T-lymphocytes as well as activated human peripheral lymphocytes contain the 1,25-$(OH)_2D_3$ receptor (Provvedini et al., 1983). In T-cells, 1,25-$(OH)_2D_3$ has recently been shown to suppress concomitantly IL-2 production and T-lymphocyte proliferation (Tsoukas et al., 1984). In all cases, the 1,25-$(OH)_2D_3$ receptor was detected by incubating intact cells with [^{3}H]1,25-$(OH)_2D_3$, isolating nuclei, and high-salt nuclear extract was used as a source of receptors.

At this point, it is not clear whether 1,25-$(OH)_2D_3$ and its receptor play a fundamental maturational role in numerous cell types, including those of the monoblastic and lymphoblastic lineages, or whether the presence of receptors in the participants of the cell-mediated immune system is part of the function of 1,25-$(OH)_2D_3$ in osteoclastic bone resorption. It is known that monocytic cells and macrophages are precursors of the bone-resorbing osteoclasts. Based upon

receptor presence, 1,25-$(OH)_2D_3$ not only participates in macrophage differentiation and the creation of osteoclast precursor, but the hormone also appears to act on T-lymphocytes which are known to produce a variety of lymphokines, including a potent bone resorbing agent known as oteoclast activating factor (Horowitz et al., 1984). Therefore, it is probably that by acting upon macrophage precursors and T-cells, 1,25-$(OH)_2D_3$ orchestrates the appearance of osteoclasts in bone. This mechanism accounts for the fact that 1,25-$(OH)_2D_3$ is the most potent bone resorbing agent known. The role of 1,25-$(OH)_2D_3$ in cell differentiation may extend to many cells containing the receptor. Moreover, the other bone mobilizing calcemic hormone, PTH, also has receptors on T-lymphocytes (Yamamoto et al., 1983).

Finally , it has been reported that 1,25-$(OH)_2D_3$ elicits the production of a T-cell derived lymphokine capable of fusing lung macrophages into giant multinucleated cells (Abe et al., 1983). These data provide a dramatic link between the operation of 1,25-$(OH)_2D_3$ on monocytes and lymphocytes: the sterol apparently initiates the differentiation of osteoclast precursor cells, a process which is then completed by the 1,25-$(OH)_2D_3$-elicited fusion factor derived from T-lymphocytes. 1,25-$(OH)_2D_3$ accentuates the production of monocytes and macrophages; the sterol is also competent in directly stimulating fusion of macrophages into multinucleate giant cells (Abe et al., 1983). In addition, 1,25-$(OH)_2D_3$ acts upon T-lymphocytes to augment IL-3 (Hodler et al., 1984) and suppress IL-2 synthesis (Tsoukas et al., 1984), as well as enhance the release of other relevant lymphokines, such as macrophage fusion factor (Abe et al., 1983).

The net result of these events is increased differentiation of precursor cells to macrophages and the creation of multinucleate cells that presumably resorb bone.

The calcemic hormone PTH is proposed to operate exclusively execute this scenario of bone resorption. A final feature of this model is the release from macrophages of the IL-1 monokine that further modifies T-lymphocytes and can act independently to resorb bone (Gowen et al., 1983). It has been demonstrated that 1,25-$(OH)_2D_3$ induced production of an intra cellular IL-1 activity in HL-60 cells (Spear et al., 1988).

PRESENCE OF 1,25-$(OH)_2D_3$ RECEPTORS IN TUMOR CELLS

Cancer cells as models of normal cells are widely used and well established. These cell lines can be considered as model systems in the same way that broken cell preparations are used for models of human physiology. Although human cancer cell studies can be criticized as unphysiological and the cells themselves as aberrant, the use of these cancer cells allows experiments that could never be done ethically in man and complements the information obtained from those experiments that can be carried out *in vivo*.

1,25-$(OH)_2D_3$ is known to elicit a number of responses in cultured cells, including inhibition of monolayer growth and morphological alteration. 1,25-$(OH)_2D_3$ has been shown to suppress the proliferation of malignant melanoma cells (Colston et al., 1981). Studies have also revealed that 1,25-$(OH)_2D_3$ functions to induce differentiation of leukemia cells to macrophages (Abe et al., 1981; Mangelsdorf et al., 1984) and prolongs the survival time of nude mice inoculated with M1 leukemia cells (Homna et al., 1983). 1,25-$(OH)_2D_3$ suppresses the growth and inhibits the malignant phenotype of rat osteosarcoma cells ROS 17/2.8 and ROS 24/1 as evidenced by a less transformed spindle-like morphology and inhibition of colony formation (Dokoh et al., 1984). Furthermore, of more than

200 surgically obtained human breast cancers examined (Eisman et al., 1981 a,b, 1981, 1982) more than half overall were 1,25-$(OH)_2D_3$ receptor positive with relatively low receptor concentrations (0.3-20 fmol/mg protein). The reason for these low receptor concentrations may be as follows: 1) the instability of the unoccupied receptor, 2) the poor exchange of labeled 1,25-$(OH)_2D_3$ onto occupied receptor and 3) the well-known heterogeneity of cancers, which comprise mixtures of cancer cells with non-malignant stromal and inflammatory cells. The presence of receptor, even with the "classical" 1,25-$(OH)_2D_3$ receptor physiochemical characteristics, in broken cell preparations of human breast cancer cell lines that have been established in culture, does not prove a role for 1,25-$(OH)_2D_3$ in human breast cancer in general.

1,25-$(OH)_2D_3$ affect the ability of a broad spectrum of malignant cell types, including several human lines, to form colonies in soft agar. Anchorage-independent growth is a recognized assay for malignant transformation, since growth of cells in agar has been significantly correlated with tumorigenicity *in vivo* (Reid, 1979). A marked, dose-dependent inhibition of colony formation was observed in the presence of 1,25-$(OH)_2D_3$, which displayed an ED_{50} of 2×10^{-10} M. This ED_{50} is equivalent to the normal circulating level of 1,25-$(OH)_2D_3$ in rodents (Hughes et al., 1975) and suggests the involvement of the 1,25-$(OH)_2D_3$ receptor in this effect. Also these same cancer cells were analyzed for their potential to form colonies in soft agar and for the number of 1,25-$(OH)_2D_3$ receptors. There was a positive correlation between receptor number and inhibition of colony formation by 10 nM 1,25-$(OH)_2D_3$. These data revealed that 1,25-$(OH)_2D_3$ is active in suppressing anchorage-independent growth in a variety of cancer cells, including melanoma, breast and bladder carcinoma and osteosarcoma.

Therefore, it has been proposed that 1,25-$(OH)_2D_3$ acts on tumor cells to inhibit colony formation, perhaps by attenuating the malignant phenotype in concert with its antiproliferative effects. The mechanism by which 1,25-$(OH)_2D_3$ functions in this system probably involves receptor binding to DNA, event which apparently alters transcription and may initiate the program for differentiation of transformed cells. Another possibility is the suppression of oncogene expression by the 1,25-$(OH)_2D_3$-receptor complex, as recently reported for c-myc in the HL-60 cell line (Reitsma, 1983). On the other hand, since 1,25-$(OH)_2D_3$ is a known calcium transport hormone, it may be the alteration of intracellular calcium that triggers cell differentiation and causes the apparent reversion of malignancy.

REGULATION OF 1,25-$(OH)_2D_3$ RECEPTORS IN TARGET CELLS

There has been some effort to learn about the possible physiological regulation of receptor number. In endocrine cells, there is abundant evidence that hormone receptor regulation can occur by two general mechanisms, agonist-specific or homologous regulation and agonist-nonspecific or heterologous regulation. Homologous refers to that form of regulation in which the hormone modulates the level of its own receptors. Conversely, heterologous indicates that exposure of the cell to one hormone alters the response to another hormone or agonist by modulating their receptor level.

For 1,25-$(OH)_2D_3$ receptors, both homologous and heterologous forms of regulation have been demonstrated *in vivo* and *in vitro*. Glucocorticoids increase 1,25-$(OH)_2D_3$ specific binding activity *in vivo* in rat and dog intestine (Hirst and Feldman, 1982a; Korkor et al., 1985) as well as in an intestinal organ culture of the rat (Massaro et al., 1982). By contrast, glucocorticoid administration reduces the

apparent intestinal content of 1,25-$(OH)_2D_3$ receptors in the mouse (Hirst and Feldman, 1982b) and in primary cultures of mouse osteoblast-like bone cells (Chen et al., 1982). Retinoic acid caused a reduction in the level of 1,25-$(OH)_2D_3$ receptors in rat osteoblast-like cells while increasing the receptor level in mouse osteoblast-like cells (Chen and Feldman, 1985) and rat osteosarcoma cells (Petkovich, 1984). The level of 1,25-$(OH)_2D_3$ receptors undergoes homologous regulation by 1,25-$(OH)_2D_3$ in a cultured kidney cell line, breast cancer cells, and rat osteosarcoma cells (Costa et al., 1985; Sher et al., 1985; Pan and Price, 1987) and in the rat kidney *in vivo* (Costa and Feldman, 1986).

The modulation of receptors within cells may reflect internal mechanism to enhance or reduce the response to a ligand. In the case of cells that are undergoing differentiation , this regulation may be associated with the maturational process. Cells are known to regulate their receptor populations in response to many external and internal stimuli, including differentiation. The specific 1,25-$(OH)_2D_3$ binding activity was stimulated by other hormones during the induction of mammary functional differentiation (Mezzetti et al., 1987). There is also evidence in HL-60 cells that 1,25-$(OH)_2D_3$ can affect the number of receptors for insulin (Chaplinski et al., 1986) and phorbol esters (Martell et al., 1987). An increase in insulin binding is associated with the appearance of markers of monocytic differentiation, while decrease levels of binding occur during myeloid differentiation of HL-60 cells or a monocytic cell line, U-937 (Chaplinski et al., 1986; Rouis et al., 1985). HL-60 cells have been shown to increase the number of [^{3}H]phorbol 12,13-dibutyrate binding sites as they differentiate *in vitro* into mature granulocytes by dimethyl sulfoxide and into monocytes by 1,25-$(OH)_2D_3$ (Martell et al., 1987; Lane et al., 1986).

PURPOSE OF THE THESIS

The availability of several clonal 1,25-$(OH)_2D_3$ receptor positive cell lines should enhance our ability to ascertain the role of 1,25$(OH)_2D_3$ in cellular differentiation. In particular HL-60 cells may provide a suitable system for these investigations, especially for describing the involvement and regulation of the 1,25-$(OH)_2D_3$ receptor in the cellular differentiation process. HL-60 cells can be induced to differentiate towards either monocytic or granulocytic pathway depending on the inducers used. Among various inducers, 1,25-$(OH)_2D_3$ has been shown to be the most potent inducer of HL-60 differentiation (Miyaura et al., 1981). The action of 1,25-$(OH)_2D_3$ on HL-60 cells is also believed to be receptor mediated since a receptor for 1,25-$(OH)_2D_3$ was identified in HL-60 cells and has the same physical characteristics of the receptor in the well characterized 1,25-$(OH)_2D_3$ target tissues. Also the ability of various analogs to cause HL-60 differentiation correlates well with their binding affinity to the 1,25-$(OH)_2D_3$ receptor (Mangelsdorf et al., 1984; Tanaka et al., 1982; Ostrem et al., 1987).

However, the mechanism by which 1,25-$(OH)_2D_3$ induces the differentiation of HL-60 cells remains unknown. In order to elucidate the mechanism of action of 1,25-$(OH)_2D_3$ and the role of 1,25-$(OH)_2D_3$ receptor in the cellular differentiation, this thesis aims to examine physical/chemical characteristics of 1,25-$(OH)_2D_3$ receptors and the regulation of receptor levels in HL-60 cells.

A serum free medium was developed to culture HL-60 cells in order to study direct effect of 1,25-$(OH)_2D_3$ and absolute potencies of vitamin D_3 metabolites for HL-60 differentiation (Chapter 2). Elimination of undefined substances, hormones, and differentiation factors present in serum from culture

medium should facilitate studies assessing regulation of 1,25-$(OH)_2D_3$ receptors and 1,25-$(OH)_2D_3$ action on HL-60 cells. Serum contains a number of proteins, which could alter 1,25-$(OH)_2D_3$ pharmacokinetics and receptor dynamics. Aside from the known components contained in serum that could induce or modify HL-60 cell differentiation, such as retinoids, interferons and vitamin D_3 metabolites, some growth factors and unidentified factors present in serum may alter 1,25-$(OH)_2D_3$'s mode of action. Serum also contains biological agents, such as glucocorticoids, retinoic acid and vitamin D_3 metabolites, which have been shown to alter the 1,25-$(OH)_2D_3$ receptor level. In addition, serum can vary widely in its composition from batch to batch. The doses of 1,25-$(OH)_2D_3$ reported in the literature, which have been shown to induce phenotypic changes in HL-60 cells cultured in serum supplemented medium, are generally larger than would be predicted by the equilibrium dissociation constant for its receptor (Tanaka et al., 1982; Mangelsdorf et al., 1984). This incongruence tends to obfuscate the issue as to whether this hormone is important for normal hematopoietic maturation. Therefore, it is necessary to remove undesirable factors in serum by culturing HL-60 cells in a serum-free chemically defined medium (SFM) in order to study true effect of 1,25-$(OH)_2D_3$ and receptor dynamics in intact HL-60 cells. Cells cultured in SFM were carefully monitored for their growth rate and the capacity to differentiate to mature monocytes when treated with 1,25-$(OH)_2D_3$ as compared to those cultured in a serum containing medium (SCM). The induction of differentiation-associated parameters was assessed by measuring the lysozyme activity, nonspecific esterase enzyme activity, expression of monocytic cell surface antigens, chemiluminescent responsiveness, and reduced proliferation. Moreover, the physical/chemical

properties of 1,25-$(OH)_2D_3$ receptors were examined for cells cultured in SFM as compared to those cultured in SCM.

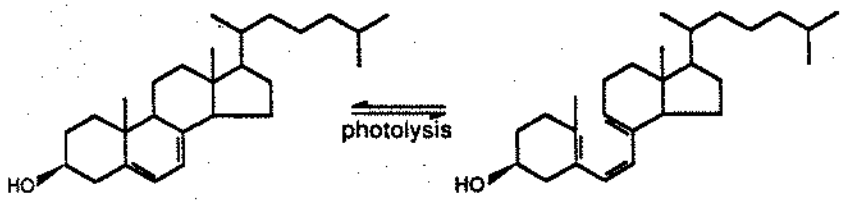
In chapter 3, several aspects of the regulation of 1,25-$(OH)_2D_3$ receptor in intact HL-60 cells by 1,25-$(OH)_2D_3$ were examined, including whether the apparent up-regulation or down-regulation is due to alterations in physical/chemical characteristics in the receptor structure, or to changes in the number of hormone binding sites. The effect of transcription or protein synthesis inhibitors on the receptor regulation and the maintenance of basal receptor level was examined using various cytotoxic agents to ascertain whether the alteration in the receptor level is mediated by nuclear-associated events.

Since these studies were performed using [^{3}H]1,25-$(OH)_2D_3$ binding activity, the potentially nonhormone binding or unoccupied forms of receptors cannot be studied. The availability of monoclonal antibody enables the study of this protein independent of hormone binding activity. The homologous regulation of 1,25-$(OH)_2D_3$ receptors in HL-60 cells was examined using monoclonal antibodies raised against the porcine intestinal 1,25-$(OH)_2D_3$ receptors (chapter 4).

Chapter 5 describes the regulation of 1,25-$(OH)_2D_3$ receptor by various differentiation inducing agents of HL-60 cells. After the bipotent HL-60 cells were incubated with known monocytic inducers or granulocytic inducers, the hormone binding activity was measured. The regulation of 1,25-$(OH)_2D_3$ receptors in intact HL-60 cells seems to be associated with monocytic differentiation as indicated by specific hormone binding activity as well as the data from immunodetection experiments.

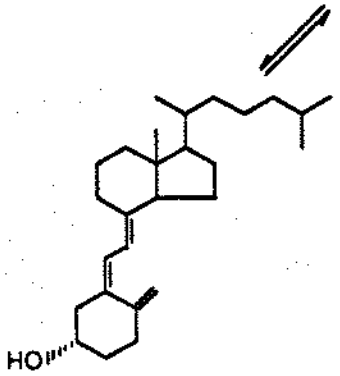
In Chapter 6, the important points resulting from this thesis research are discussed in terms of how they fit into the already present body of literature on the role of 1,25-$(OH)_2D_3$ and its receptor in the HL-60 cell differentiation.

Figure 1. Photolysis of 7-dehydrocholesterol in skin to produce vitamin D_3.



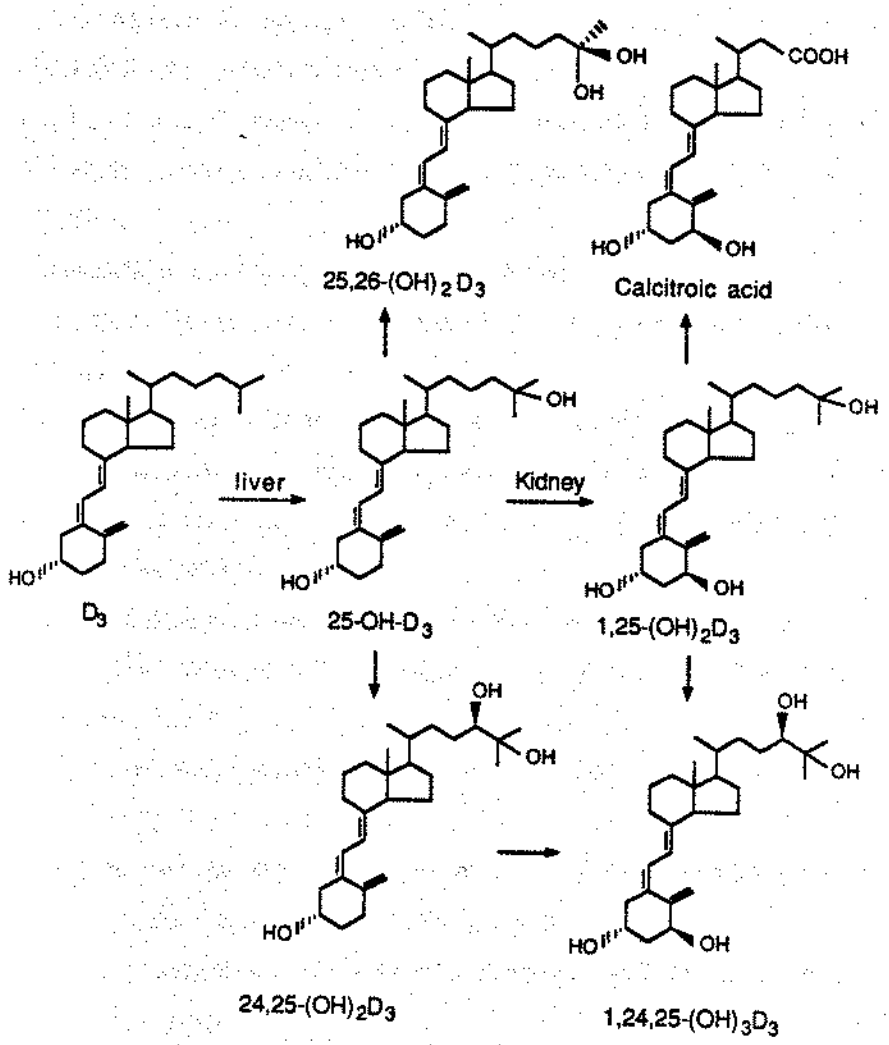
7-dehydrocholesterol

Pre vitamin D_3



Vitamin D_3

Figure 2. Metabolism of vitamin D to its known metabolites.


OH
OH
HO
25,26-(OH)2D3
COOH
HO
OH
Calcitroic acid
liver
Kidney
HO
D3
OH
HO
25-OH-D3
OH
HO
OH
1,25-(OH)2D3
OH
OH
HO
24,25-(OH)2D3
OH
OH
HO
OH
1,24,25-(OH)3D3

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CHAPTER II

INDUCTION OF MONOCYTIC DIFFERENTIATION BY CALCITRIOL (1,25-DIHYDROXYVITAMIN D_3) IN THE HUMAN PROMYELOCYTIC LEUKEMIC CELL LINE (HL-60) IN SERUM-FREE MEDIUM

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(See Appendix)

CHAPTER III

RECEPTORS FOR 1,25 DIHYDROXYVITAMIN D_3 UNDERGO HOMOLOGOUS REGULATION IN HUMAN PROMYELOCYTIC CELLS (HL-60)

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SUMMARY

The regulation of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) receptors in intact human promyelocytic leukemic cells, HL-60 cells, cultured in serum-free conditions was investigated in association with hematopoietic cellular differentiation. Receptors in intact HL-60 cells appeared to be saturated with [^{3}H]1,25-$(OH)_2D_3$ within 1 h after hormone addition at 37° C. Continuous exposure of cells to hormone for longer times resulted in an apparent up-regulation (increase in specifically bound [^{3}H]1,25-$(OH)_2D_3$) which was maximal between 8-16 h after hormone addition. This was followed by an apparent down-regulation (loss of specifically-bound [^{3}H]1,25-$(OH)_2D_3$) which could be observed by 24 h. Scatchard analysis performed at 2, 12, and 24 h after hormone addition indicated that there was a single class of binding sites with similar apparent dissociation constants (K_d). Data from sucrose density gradient analysis, DNA-cellulose chromatography and fast protein liquid chromatography (FPLC) indicated no significant changes in these three receptor groups. However, the estimated number of binding sites had increased two-fold by 14 h followed by a decrease to basal receptor level by 24 h after addition of hormone to cells. The up-regulation was completely inhibited by inhibitors of transcription, actinomycin D (2×10^{-6} M), ethidium bromide (10^{-4} M) or α-amanitin (2×10^{-4} M) and an inhibitor of translation, cycloheximide (10^{-5} M). Both the monocyte cell line U937 and macrophage-like cell line P388D1 could up- and down-regulate the 1,25-$(OH)_2D_3$ receptors upon continuous exposure to 1,25-$(OH)_2D_3$. Taken together, these data indicate that 1,25-$(OH)_2D_3$ receptors undergo up-regulation followed by down-regulation in intact human hematopoietic cells and this alteration in receptor level

may be one of the earliest phenotypic changes that occurs in HL-60 cells stimulated towards the monocytic pathway.

INTRODUCTION

Receptors for 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), the biologically active metabolite of vitamin D_3, have been identified in hematopoietic cells including normal human lymphocytes and monocytes [1,2] as well as several clonal cell lines with monocytic and myeloid characteristics [3-6]. The biological responses to 1,25-$(OH)_2D_3$ in these target cells are believed to be mediated by the binding of 1,25-$(OH)_2D_3$ to intracellular receptors which consequently modulate gene expression [7-9]. 1,25-$(OH)_2D_3$ has been shown to promote *in vitro* differentiation of normal bone marrow cells [10], peripheral human monocytes [11], and to regulate directly macrophage function [12]. Moreover, there are several reports that monoblastic and myeloblastic cell lines [13-15], including the human promyelocytic HL-60 cell line [4,10], are differentiated into macrophages in the presence of 1,25-$(OH)_2D_3$.

Several lines of evidence exist which implicate the 1,25-$(OH)_2D_3$ receptor in mediating 1,25-$(OH)_2D_3$ induction of monocytic differentiation. The existence of a positive correlation between 1,25-$(OH)_2D_3$-induced differentiation and the occurrence of occupied 1,25-$(OH)_2D_3$ receptors has been reported in HL-60 cells [4]. The functional defect of 1,25-$(OH)_2D_3$ resistant HL-60 clones has been correlated with a reduced amount of specific 1,25-$(OH)_2D_3$ receptors [16]. Lastly, the rank order of vitamin D_3 metabolites inducing monocytic differentiation is similar to their affinity to the 1,25-$(OH)_2D_3$ receptor in HL-60 cells [3,17].

Receptor presence for 1,25-$(OH)_2D_3$ sensitive target cells has been correlated also with the magnitude of the biological response. For instance, 1,25-$(OH)_2D_3$-induced changes in morphology and regulation of growth in several rat osteogenic sarcoma cell lines were dependent on the number of receptor molecules

per cell [18]. Cultured kidney cells with reduced 1,25-$(OH)_2D_3$ receptors exhibited a diminished capacity to respond to 1,25-$(OH)_2D_3$ as measured by 24-hydroxylase activity [19]. The magnitude of 24-hydroxylase activity and/or inhibition of collagen synthesis has been correlated directly with the abundance of receptors in cultured mouse osteoblast-like cells [20-22]. Data presented by Haussler and coworkers [23] have shown an interdependent relationship between 1,25-$(OH)_2D_3$ receptor number and inhibition of colony formation by 1,25-$(OH)_2D_3$.

In endocrine target cells, there is abundant evidence that hormone receptor regulation can occur by two general mechanisms, agonist-specific or homologous regulation and agonist-nonspecific or heterologous regulation. Homologous refers to that form of regulation in which the hormone modulates the level of its own receptors. Conversely, heterologous indicates that exposure of the cell to one hormone alters the response to another hormone or agonist by modulating their receptor levels. At least for 1,25-$(OH)_2D_3$ receptors, both homologous and heterologous forms of regulation have been reported *in vivo* and *in vitro*. Glucocorticoids increase 1,25-$(OH)_2D_3$ specific binding activity *in vivo* in rat and dog intestine [24,25] as well as in an intestinal organ culture of the rat [26]. By contrast, glucocorticoid administration reduces the apparent intestinal content of 1,25-$(OH)_2D_3$ receptors in the mouse [27] and in primary cultures of mouse osteoblast-like bone cells [28]. Retinoic acid caused a reduction in the level of 1,25-$(OH)_2D_3$ receptors in rat osteoblast-like cells while increasing the receptor level in mouse osteoblast-like cells [22] and rat osteosarcoma cells [29]. The level of 1,25-$(OH)_2D_3$ receptors undergoes homologous regulation by 1,25-$(OH)_2D_3$ in a cultured kidney cell line, breast cancer cells, and rat osteosarcoma cells [30-32] and in the rat kidney *in vivo* [33]. There is also evidence in HL-60 cells that 1,25-

$(OH)_2D_3$ can affect the number of receptors for insulin [34] and phorbol esters [35].

The modulation of receptors within cells may reflect an internal mechanism to enhance or reduce the response to a ligand; in the case of cells that are undergoing differentiation, this regulation may be associated with the maturational process. In the present studies, we have attempted to ascertain whether 1,25-$(OH)_2D_3$ receptors in HL-60 cells are subject to homologous regulation. HL-60 cells were cultured in a serum-free chemically-defined medium to examine more precisely 1,25-$(OH)_2D_3$ induced changes without the influence from the myriad number of factors contained within serum [36]. The data presented indicate that 1,25-$(OH)_2D_3$ receptors undergo apparent homologous up-regulation followed by down-regulation as defined by [^{3}H]1,25-$(OH)_2D_3$ binding.

MATERIALS AND METHODS

Materials. 1,25-$(OH)_2D_3$, radiolabeled with tritium at the 26,27-methyl groups ([^{3}H]1,25-$(OH)_2D_3$, 176-180 Ci/mmol), was obtained from the Radiochemical Center (Amersham, Buckinghamshire, U.K.). Nonradioactive 1,25-$(OH)_2D_3$ was a gift from Dr. M. Uskokovic of Hoffmann-LaRoche, Inc. (Nutley, NJ). Proteinase K, and α-amanitin were purchased from Sigma (St. Louis, MO), hydroxylapatite from BioRad Laboratories (Richmond, CA). PBS was purchased from GIBCO (Grand island, NY), and fetal calf serum from Hyclone (Logan, UT). All other reagents were of analytical grade.

Cell Culture. The human promyelocytic leukemic cells, HL-60, were obtained from American Type Tissue Culture (Bethesda, MD) and were maintained

in RPMI-1640 medium buffered with 25 mM Hepes (Sigma Chemical Co., St. Louis, MO) containing 100 units/ml penicillin and 0.5 μg/ml fungizone. This basal medium was supplemented with a defined supplement of insulin from bovine pancreas (5 mg/ml), human transferrin (5 mg/ml), sodium selenite (5 ng/ml), and BSA (fatty acid free, 0.5 mg/ml) (Sigma Chemical Co., St. Louis, MO), designated as serum free medium (SFM). HL-60 cells were grown in SFM at 37° C in a humidified 95% air-5% CO_2 atmosphere and subcultured with 4 day intervals at a seeding density of 3×10^5 cells per ml.

A human monocyte cell line, U937 [14], and a mouse macrophage-like cell line, P388D1 [15], were cultured in the same basal medium supplemented with heat-inactivated (56° C for 30 min) 10% (v/v) fetal bovine serum, designated as 10% serum containing medium (SCM). Adherent cells, P388D1, were removed from the flasks by scraping them gently. Cell proliferation was assessed by counting cells in a hemocytometer, and cell viability was determined by trypan blue dye exclusion.

Binding Studies. Total cellular 1,25-$(OH)_2D_3$ receptor concentration was quantitated as previously described [36]. HL-60 cells grown in SFM were rinsed and resuspended at a density of 2 to 5×10^6 cells per ml of SFM. Cells were labeled with various concentrations of [^{3}H]1,25-$(OH)_2D_3$ in the presence or absence of a 200-fold excess of 1,25-$(OH)_2D_3$ for various times at 37° C in a humidified 95% air-5% CO_2 atmosphere. For U937 and P388D1 cells grown in 10% SCM, cells were labeled with [^{3}H]1,25-$(OH)_2D_3$ in 1% SCM. At the end of incubation periods, cells were washed two times with PBS and resuspended in icecold TEDM for 20 min with intermittent vortexing. Receptor-bound [^{3}H]1,25-$(OH)_2D_3$ was quantitated by HAP batch assay [37] with slight modification [36].

The radioactivity was quantitated by liquid scintillation spectrometry in a Packard Tri-Carb spectrometer (model Prias PLD) using Opti-Fluor scintillation fluid (Packard, Downers Grove, IL) with an efficiency for tritium of 30%. The concentration of 1,25-$(OH)_2D_3$ binding sites and apparent equilibrium dissociation constant (K_d) were derived by the method of Scatchard [38].

Preparation of 1,25-$(OH)_2D_3$ Receptors. 1,25-$(OH)_2D_3$ receptors were prepared as described previously [36]. Briefly, HL-60 cells (50×10^6) cultured in SFM were rinsed and resuspended in 2 ml of SFM. After cells were labeled with 1 nM [^{3}H]1,25-$(OH)_2D_3$ in the presence or absence of 200-fold excess of 1,25-$(OH)_2D_3$ for various times at 37° C in a humidified 95% air-5% CO_2 atmosphere, cells were washed two times in PBS followed by centrifugation at 500 g for 5 min. The following manipulations were carried out between 0° and 4° C. After cells were resuspended and incubated for 20 min in TEDM buffer with intermittent vortexing, cells were homogenized using a Tissuemizer type SDT (Tekmar Co., Cincinnati, OH) for 3 sec at a speed setting of 50. The homogenate was centrifuged for 10 min at 800 g and the pellet was designated as a crude nuclear fraction. The crude nuclear pellet was incubated with TEDMK-0.3 for 30 min followed by centrifugation at 105,000 g for 45 min in a Beckman L5-50B ultracentrifuge using a type Ti-50 rotor (Beckman Instruments, Palo Alto, CA). The supernatant was utilized as the source of [^{3}H]1,25-$(OH)_2D_3$-labeled nuclear receptor for physical/chemical characterization.

Physical/Chemical Characterization of 1,25-$(OH)_2D_3$ Receptor. Sucrose density gradient analysis and DNA-cellulose chromatography were performed as described previously [36]. To perform the fast protein liquid

chromatography (FPLC) of [^{3}H]1,25-$(OH)_2D_3$ labeled nuclear receptor, nuclear extract was diluted tenfold (v/v) with TEDM to reduce ionic strength (i.e., to a final concentration of KCl equal to 30 mM). Aliquots of diluted receptors were injected onto an analytical strong anion exchange column (MonoQ®) (0.5 × 5 cm). Elution of receptor was carried out by employing a linear KCl gradient (0.03-0.4 M). Fractions (1.0 ml) were collected and 0.5 ml aliquots were removed to determine the radioactivity.

Measurement of mRNA and Protein Synthesis. Total amount of mRNA was measured by Quick-blot technique [39] with slight modification. Briefly, control and cytotoxic agent treated cells (5 x 10^6 cells in 1 ml of SFM) were incubated with 5 μCi of [^{3}H]uridine (39.6 Ci/mmol, Dupont, Boston, MA) for various times at 37° C in a humidified 95% air-5% CO_2 atmosphere. At the end of incubation periods, cells were washed two times with HBSS and resuspended in 0.5 ml of HBSS containing a ribonuclease inhibitor, vanadylribonucleoside complexes. Cells then were incubated with proteinase K at a final concentration of 200 mg/ml in 37° C water bath for 30 min. After cells were mixed with 1/20 vol of 10% Nonidet P-40 and 10% sodium-deoxycholate, one volume of supersaturated NaI was added. Aliquots were filtered through nitrocellulose membranes under vacuum and the filters were soaked in RNase free water and 70% ethanol. The dried filters were counted to determine radioactivity by liquid scintillation spectrometry. In order to facilitate the penetration of α-amanitin into HL-60 cells, cells were electroporated [40]. HL-60 cells (5 × 10^6) suspended in ice-cold SFM with or without α-amanitin were placed into a cell chamber (Prototyp Design Services, Madison, WI) of length 5 mm and crosssectional area of 1 cm^2 at 0° C. A bank of capacitors charged to 1 KV was discharged through the sample via an

electronic switch (PDS model ZA1000, Madison, WI). (From preliminary studies, 1 KV was determined to be the optimum voltage which permitted entry of *a*-amanitin and cell viability of over 80%.) After electroporation, cells were placed on ice for 10 min and then kept at 37° C humidified 95% air-5% CO_2 atmosphere for an hour before further experiments were performed. Cell viability was determined by trypan blue exclusion. Protein synthesis was estimated by measuring [^{3}H]leucine incorporation (53 Ci/mmol, Amersham, Buckinghamshire, U.K.).

Statistical Analysis. Where appropriate, statistical comparisons of the means of independent samples were made using Student's t-test.

RESULTS

A time course for the regulation of [^{3}H]1,25-$(OH)_2D_3$ uptake into intact HL-60 cells is shown in Fig. 1. Receptors in intact HL-60 cells grown in SFM appear to be saturated with using 1 nM [^{3}H]1,25-$(OH)_2D_3$ within 1 h after addition of [^{3}H]1,25-$(OH)_2D_3$. Previously, we have demonstrated under similar conditions that HL-60 cell 1,25-$(OH)_2D_3$ receptors became saturated by exposing them to concentrations of 1,25-$(OH)_2D_3$ greater than 0.8 nM [36]. Continuous exposure of cells to hormone for longer times resulted in an apparent "up-regulation" (increase in specifically bound [^{3}H]1,25-$(OH)_2D_3$). The amount of specific binding became maximal between 8 and 16 h, a time when binding was typically 2- to 3-fold higher than the 1 h time period (e.g. basal level). This phase is followed by apparent "down-regulation" (loss of specifically bound [^{3}H]1,25-$(OH)_2D_3$) which could be observed by 24 h. The experiment shown in Fig. 2 was performed to eliminate, in part, the possibility that metabolism of 1,25-$(OH)_2D_3$ was responsible for the loss

of specific binding observed between 24 and 48 h. The addition of a second dose of [^{3}H]1,25-$(OH)_2D_3$ to HL-60 cells at 24 h failed to label receptors at a level commensurate with the maximum level.

A second set of experiments was conducted to eliminate the possibility that the apparent "down regulation" of receptors represented losses due to inflated nonspecific binding during the longer incubation times. The nonspecific binding for cells incubated in the presence of 200-fold excess of nonradioactive 1,25-$(OH)_2D_3$ remained relatively constant throughout the time course of the experiments. For instance, the nonspecific binding estimates were 13.2 ± 2.9, 12.0 ± 3.6 and 17.8 ± 5.4% of the total binding for the cells incubated with 1.0 nM [^{3}H]1,25-$(OH)_2D_3$ in the presence or absence of a 200-fold excess nonlabeled hormone for 2, 12, 24 h, respectively, and the values represent the mean ± SE for an average of 8 separate experiments. To further confirm the constancy of nonspecific binding values, cells were incubated with 1.0 nM [^{3}H]1,25-$(OH)_2D_3$ for 2 or 12 h at 37° C as described previously. Nuclear receptors were obtained via salt extraction; portions were incubated at 30° C with a 200-fold excess of nonradioactive 1,25-$(OH)_2D_3$ to displace the bound [^{3}H]1,25-$(OH)_2D_3$ and used as a measure of nonspecific binding. Figure 3 shows that rapid displacement of bound [^{3}H]1,25-$(OH)_2D_3$ with nonradioactive hormone (filled symbols) occurred at 30° C from cells that had been labeled for either 2h (Δ) or 12 h (O). Moreover, the nonspecific binding estimates determined in the presence of excess nonradioactive 1,25-$(OH)_2D_3$ at 1 or 2 were similar and represented approximately 9% of the total binding.

The effect of cell density on the regulation of 1,25-$(OH)_2D_3$ receptors also was investigated (Fig. 4). Cells seeded at densities ranging from 5×10^5 to $5 \times$

10^6 cells/ml were incubated in the presence 1.0 nM [^{3}H]1,25-$(OH)_2D_3$ for various times. The apparent up- and down-regulation of 1,25-$(OH)_2D_3$ receptors occurred at all seeding densities, although down-regulation did not occur as rapidly in the cells seeded at 5×10^5 cells/ml.

To ascertain whether the alterations in hormone binding were due to the changes in number of binding sites or affinity for hormone, binding was analyzed by the method of Scatchard (Fig. 5). The apparent equilibrium dissociation constants (K_d's) determined at 2 (Panel A), 14 (Panel B), and 24 h (Panel C) after addition of hormone were not significantly different ($P > 0.1$) and the analysis indicated only one population of binding sites. However, the estimated number of binding sites had increased 2-fold by 14 h, followed by decrease in the number of binding sites to the basal level by 24 h (see insets in Fig. 5). The alterations in the level of hormone binding were further characterized by sucrose density gradient analysis (Fig. 6). The extracts from cells previously treated with [^{3}H]1,25-$(OH)_2D_3$ for 2, 12, and 24 h were layered on linear 4-20% sucrose gradients. The results showed a single peak in all three groups of receptors sedimenting at 3.4 S, similar to that of 1,25-$(OH)_2D_3$ receptors characterized in several systems [41]. The inherent ability of 1,25-$(OH)_2D_3$ receptors to bind to DNA-cellulose was examined (Fig. 7). The 1,25-$(OH)_2D_3$ receptors from HL-60 cells treated with hormone for 2, 12, and 24 h were applied to a DNA-cellulose column and eluted with a buffered salt gradient, 0.03-0.5 M KCl. Receptors from each of the three treatment times were retained under the low ionic strength and eluted at a KCl concentration of 0.18 M. Basal, up-regulated, and down-regulated receptors were characterized further by FPLC.

inhibi The results presented in Figure 9 demonstrate that the amount of specific $[^3H]1,25\text{-}(OH)_2D_3$ binding, or concentration of occupied receptors, as a function of time is dependent upon hormone concentration. Within the range of doses tested, maximal binding occurred in the presence of 0.8 nM $[^3H]1,25\text{-}(OH)_2D_3$ and the time at which maximal binding was observed was similar for each dose (e.g. ~12 h). To separate the contribution of the time necessary for different doses of $1,25\text{-}(OH)_2D_3$ to occupy and saturate receptors from the dose-sensitive induction of receptors (i.e. "up-regulation"), cells first were incubated with various doses of $[^3H]1,25\text{-}(OH)_2D_3$ for 12 h and then incubated additionally with a saturating dose of $[^3H]1,25\text{-}(OH)_2D_3$ for 1 h. As depicted in Figure 10, incubation of cells for 12 h with increasing concentrations of $[^3H]1,25\text{-}(OH)_2D_3$ (0.01-1.5 nM) resulted in the expected rises in receptor occupancy (hatched bars). To quantitate the total amount of receptor present for each given dose at the end of 12 h induction period, a second incubation with 1.0 nM $[^3H]1,25\text{-}(OH)_2D_3$ was employed to fill any remaining unoccupied receptors (open bars). When compared to the basal level of receptors (2 h; solid bar), doses between 0.05-1.5 nM resulted in a dose-dependent "up-regulation" of receptors under conditions where receptors were fully occupied (open bars). As expected, those concentrations of $[^3H]1,25\text{-}(OH)_2D_3$ that were nearly saturating or saturating (0.6 and 1.5 nM, respectively) did not show large increases in specific binding upon the second exposure of hormone (Fig. 10, open vs. hatched bars). re 12. The inhibitors of transcription, actinomycin D, ethidium

To determine whether this apparent regulation of $1,25\text{-}(OH)_2D_3$ receptors is dependent on transcriptional and/or translational events, several cytotoxic agents were employed to inhibit mRNA synthesis or protein synthesis. Figure 11A shows the dose-response inhibition of transcription by actinomycin D, with maximum

labeled with [^{3}H]1,25-$(OH)_2D_3$ for 2 h to measure the amount of receptors, unoccupied receptor level fell with a shorter half life of 2.5 h. Control cells subjected to electroporation still exhibited the increase in hormone binding activity of the receptor in a similar fashion to control cells. Moreover, electroporesed cells at 1 KV still were able to respond to 1,25-$(OH)_2D_3$ and differentiate towards the monocytic pathway (data not shown). The inhibitor of protein synthesis, cycloheximide, also inhibited the increase in hormone binding activity, but had no apparent effect on receptor down-regulation at 24 and 48 h when cycloheximide was added to cells at 12 h (Fig. 12B). In contrast, cells treated concomitantly with cycloheximide and [^{3}H]1,25-$(OH)_2D_3$ at time zero showed minimal measurable receptor loss compared to the loss that was observed in cells treated with transcriptional inhibitors. The cells pretreated with cycloheximide for up to 24 h and labeled with [^{3}H]1,25-$(OH)_2D_3$ for 2 h also showed a slower decrease in receptor level than those cells pretreated with actinomycin D. This concentration of cycloheximide inhibited protein synthesis by about 92% as measured by [^{3}H]leucine incorporation and had no deleterious effect on cell viability (data not shown). The basal receptor level from cells treated with vehicle was not significantly altered for up to 24 h (data not shown).

In order to generalize the findings that regulation of 1,25-$(OH)_2D_3$ receptors might be correlated with the monocytic, differentiation pathway, other cell lines with monocyte/macrophage characteristics were examined for their ability to regulate 1,25-$(OH)_2D_3$ receptor level. Both the human monocytic cell line, U937, and mouse macrophage-like cell line, P388D1, exhibited similar alterations in receptor level (Fig. 13). In U937 cells, induction of the increase in hormone binding occurred more rapidly than that in HL-60 cells. The maximum level of

hormone binding was observed between 6-8 h after addition of hormone to the culture, followed by loss of hormone binding. P388D1 cells exhibited a similar time-course of alterations in receptor level to those in HL-60 cells.

DISCUSSION

Our experimental results provide evidence that 1,25-$(OH)_2D_3$ autoregulates its own receptor in the HL-60 cell. The incubation of intact cells with 1-2 nM [^{3}H]1,25-$(OH)_2D_3$ resulted in saturation of 1,25-$(OH)_2D_3$ receptors within 1 to 2 h after hormone addition (Fig. 1) (36). Continuation of exposure to hormone resulted in a further accumulation of specific 1,25-$(OH)_2D_3$ binding which became maximal between 8 and 16 h. This slower phase of rise in hormone binding appears to be due to an induction event which is presumed to be mediated by nuclear effects. Previous results have demonstrated that 1,25-$(OH)_2D_3$ entry into intact human cancer cells in culture is very rapid and reaches equilibrium with medium concentrations within 15 sec [31]. Therefore, the rapid entry of 1,25-$(OH)_2D_3$ into cultured cells could not explain the slower phase of [^{3}H]1,25-$(OH)_2D_3$ binding in HL-60 cells. Moreover, it is unlikely that it requires about 12 h to saturate the existing receptors considering the high affinity of 1,25-$(OH)_2D_3$ receptors for its ligand (~10^{-11}-10^{-10} M). This initial phase of binding also could not be influenced by endogenous 1,25-$(OH)_2D_3$ exchange as these cells have been passaged in SFM for 5-10 passages prior to the initiation of experiments. Essentially the 1,25-$(OH)_2D_3$ receptor population in these HL-60 cells is virtually ligand free (e.g. exists in an unoccupied state). Although receptor occupancy itself could lead to increased receptor stability and decreased receptor degradation [30], the slow phase of receptor rise might not be explained by this mechanism due to the

relative long time required to reach a maximal response. It has been suggested that vitamin D_3 metabolites up-regulated the number of 1,25-$(OH)_2D_3$ receptors by a receptor-mediated induction event in a cultured kidney cell line [30]. Mutant skin fibroblasts from patients with vitamin D-dependent rickets type II [42], containing nonresponsive 1,25-$(OH)_2D_3$ receptors because of a defect in DNA binding, also failed to exhibit the characteristic up-regulation observed in normal cells [30]. More recently, it has been demonstrated that 1,25-$(OH)_2D_3$ can increase mRNA activity for its receptor in 3T6 cells, indicative of receptor autoregulation [43]. These experimental results support our observation of homologous up-regulation of 1,25-$(OH)_2D_3$ receptors in a 1,25-$(OH)_2D_3$ target cell.

Since homologous regulation of 1,25-$(OH)_2D_3$ receptors was being studied in intact cultured cells by analysis of [^{3}H]1,25-$(OH)_2D_3$ binding, we wanted to ensure that nonspecific binding estimates were not variable and did not influence the interpretation of results. For example, if nonspecific binding were to rise throughout the time course of the experiment, the extent of down regulation could have been overestimated. However, careful analysis of our results indicated that nonspecific binding was stable throughout the culture period. Secondly, estimates of nonspecific binding carried out on extracted receptors (Fig. 3) provided similar values.

In order to examine whether up-regulation was associated with secondary binding sites and/or an additional receptor population, we characterized and quantitated up-regulated and down-regulated 1,25-$(OH)_2D_3$ receptors in comparison to basal receptors. Scatchard analysis indicated that only one binding population existed and the apparent equilibrium dissociation constants (K_d's) were not significantly changed in the up- and down-regulated receptors (Fig. 5).

However, the estimated number of receptor sites was increased 2-fold by 14 h followed by decrease in the number of receptor sites to the basal receptor level by 24 h. Moreover, the data from sucrose gradient analysis, DNA-cellulose chromatography and FPLC (Fig. 6-8) indicated that no significant changes in physical/chemical characteristics occurred with up- and down-regulated receptors.

The finding that the apparent up-regulation of 1,25-$(OH)_2D_3$ receptors in HL-60 cells was dependent on new protein and new mRNA synthesis provides additional evidence that receptor occupancy causes an induction event. The inhibitors of transcription employed, actinomycin D, ethidium bromide, are DNA-intercalating agents, which might interfere with nuclear binding sites of 1,25-$(OH)_2D_3$ receptor complex [31]. Therefore, the inhibitor of RNA polymerase II in eukaryotic cells, α-amanitin, was employed to ascertain the effect of cytotoxic agents on receptor regulation at transcriptional level. Transformed cells, as a rule, are less sensitive to α-amanitin than the corresponding normal cells probably due to the poor penetration of the drug into cells [44]. As shown in Figure 11B, a high concentration of α-amanitin (10^{-4} M) did not inhibit the transcription of the intact cells. Thus, the cells were subjected to electroporation techniques to facilitate the penetration of drug into cells. The HL-60 cells subjected to electroporation were also able to up-regulate hormone binding activity (Fig. 12A) and respond to 1,25-$(OH)_2D_3$ in a similar fashion to control cells and were morphologically indistinguishable from the control cells. It has been reported previously that only 40% of homologously induced 1,25-$(OH)_2D_3$ receptor accumulation was blocked by incubation with 10^{-5} M α-amanitin and was only effective when added prior to 6 h incubation with hormone in rat osteosarcoma cells [32]. Incomplete inhibition in

these studies might have resulted from the poor penetration of α-amanitin into the osteosarcoma cells.

The inhibitors of transcription tested lowered the basal receptor level rapidly as shown in Figure 12A. Actinomycin D did not interfere with the binding of [^{3}H]1,25-$(OH)_2D_3$ to its receptor *in vitro* (data not shown). Taken together these results suggest that the occupied 1,25-$(OH)_2D_3$ receptor is a dynamic and rapidly turning over protein with a half-life of about 3.5 h. This observation is well correlated with the relatively short half-lives of the estrogen receptor in breast cancer cells and uterine cells [45,46], the androgen receptors in the ductus deferens tumor cells [47] and 1,25-$(OH)_2D_3$ receptor in a cultured kidney cell line [48]. Cycloheximide, an inhibitor of protein synthesis, did not seem to lower the basal receptor level significantly when cells were incubated with [^{3}H]1,25-$(OH)_2D_3$ simultaneously (Fig. 12B). For the estrogen receptor, turnover has been shown to be completely arrested by incubation of uterine cells with cycloheximide [46]. Cycloheximide can also alter other protein products that might be involved in receptor degradation. Proteases which inactivate receptors also have been described in crude cytoplasmic and nuclear preparations of other steroid hormones and 1,25-$(OH)_2D_3$ [49-51]. Therefore, the observed cycloheximide-induced stabilization might be due to inhibition of protease action.

After up-regulation of 1,25-$(OH)_2D_3$ receptors has occurred, addition of cycloheximide did not alter the time course of down-regulation (Fig. 12B). This may be due to accumulation of proteins involved in receptor turnover in cells during the 12-hour 1,25-$(OH)_2D_3$ treatment. It also may be similar to the observation that 1,25-$(OH)_2D_3$ receptor processing in human breast cancer cells does not require new protein synthesis [31]. The control of the expression of steroid hormone

action at the nucleus is not well understood. Some mechanism must exist for the termination of the effect of the hormone-receptor complex. The disappearance of hormone-receptor complexes has been shown to be largely due to a loss of hormone-binding capacity of steroid hormone receptors (processing) [55,56]. We have shown that intact HL-60 cells exhibited loss of specific 1,25-$(OH)_2D_3$ binding, which can be explained by degradation of hormone and/or lost of functional receptor (i.e. processing). It has been reported that 1,25-$(OH)_2D_3$ induces vitamin D_3 metabolism in cell lines including HL-60 cells [52-53]. However, metabolism could not explain this loss in our experiments since the addition of fresh hormone at 24 h did not restore binding to the levels observed during up-regulation (Fig. 2). While these experiments relied on binding data to measure diminishing receptor levels, it is possible that other proteins might have been labeled. However, our analyses, including Scatchard analysis (Fig. 5), physical/chemical analyses (Fig. 6-8) and preliminary data of polyacrylamide gel electrophoresis using iodinated anti-receptor antibodies to detect 1,25-$(OH)_2D_3$ receptors [54], indicate that specific [^{3}H]1,25-$(OH)_2D_3$ binding is a reliable indicator of 1,25-$(OH)_2D_3$ binding to only 1,25-$(OH)_2D_3$ receptors. Therefore, this rapid loss of specific binding in the intact HL-60 cell may represent the effect of a control mechanism such as receptor processing.

An interesting consideration concerning the changes in apparent 1,25-$(OH)_2D_3$ receptor levels in HL-60 cells is that these alterations might be components of the cellular differentiation process induced by monocytic inducers. HL-60 cells have been shown to regulate other types of receptors during monocytic and granulocytic differentiation. Insulin receptor expression can be increased and decreased selectively in HL-60 cells after differentiation with monocytic and

granulocytic inducers, respectively [34]. HL-60 cells regulate their phorbol diester receptor number as they differentiate towards granulocytes [57] and monocytes [35]. The regulation of hormone receptors in association with functional differentiation also has been reported in other cell systems [58,59]. In order to generalize the findings that regulation of 1,25-$(OH)_2D_3$ receptors might be correlated with the monocytic differentiation pathway, other cell lines with monocyte/macrophage characteristics were examined. Both human monocytic cell line, U937, and mouse macrophage-like cell line, P388D1, have been reported to be 1,25-$(OH)_2D_3$ target tissues and have specific 1,25-$(OH)_2D_3$ receptors [5,14,15]. The similar alterations in 1,25-$(OH)_2D_3$ receptor level were observed in these cell lines (Fig. 13). The physiological significance of our findings that HL-60 cells may regulate 1,25-$(OH)_2D_3$ receptors as they differentiate into monocytes remains unknown. This may reflect a portion of an internal mechanism to enhance or change response to an endogenous ligand for its receptor as cells mature. Most differentiation-associated phenotypic changes in HL-60 cells appeared after at least 24 hour-treatment with 1,25-$(OH)_2D_3$, while the up-regulation of 1,25-$(OH)_2D_3$ receptors occurred between 3-12 h after exposure to 1,25-$(OH)_2D_3$. Therefore, an increase in 1,25-$(OH)_2D_3$ receptor binding may serve as an early and sensitive marker of monocytic differentiation in HL-60 cells.

In summary, we have demonstrated for the first time the up-regulation of 1,25-$(OH)_2D_3$ receptor followed by down-regulation in intact human target cells cultured in serum-free condition. Maintenance and apparent up-regulation of receptor require new mRNA synthesis. The present data suggest that 1,25-$(OH)_2D_3$ receptor regulation in intact HL-60 cells might be a component of the cellular differentiation process. Further studies concerning the biochemical basis

for 1,25-$(OH)_2D_3$ receptor regulation should lead to new understanding of the possible role of 1,25-$(OH)_2D_3$ receptors in the differentiation process and receptor dynamics.

ABBREVIATIONS

PBS, Dulbecco's phosphate buffered saline; TED, 50 mM Tris-HCl, pH 7.5, 1.5 mM EDTA, 2 mM dithiothreitol; TEDM, 50 mM Tris-HCl, pH 7.5, 1.5 mM EDTA, 5 mM dithiothreitol, 10 mM sodium molybdate; TEDMK, TEDM buffer with various concentrations of KCl (e.g., K-0.03 = 0.03 M KCl); TDK, 10 mM Tris-HCl, pH 7.5, 0.5 mM dithiothreitol, 30 mM KCl; TDT, 10 mM Tris-HCl, pH 7.5, 0.5 mM dithiothreitol, 0.1% Triton X - 100 (v/v); BSA, bovine serum albumin; SFM, serum free medium; SCM, serum containing medium; FPLC, Fast Protein Liquid Chromatography; HBSS, Hanks buffered salt solution; DFP, diisopropyl fluorophophate.

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Figure 1. Time-dependent regulation of [^{3}H]1,25-$(OH)_2D_3$ uptake into intact HL-60 cells cultured in SFM. Cells (1×10^7) were incubated at 37° C in humidified 95% air-5% CO_2 atmosphere with 1 nM [^{3}H]1,25-$(OH)_2D_3$ in the presence or absence of a 200-fold excess of unlabeled 1,25-$(OH)_2D_3$ for various times. Specifically bound [^{3}H]1,25-$(OH)_2D_3$ was quantitated by the HAP batch assay. Values represent the mean for two to four replicate wells with duplicate measurements from each well.

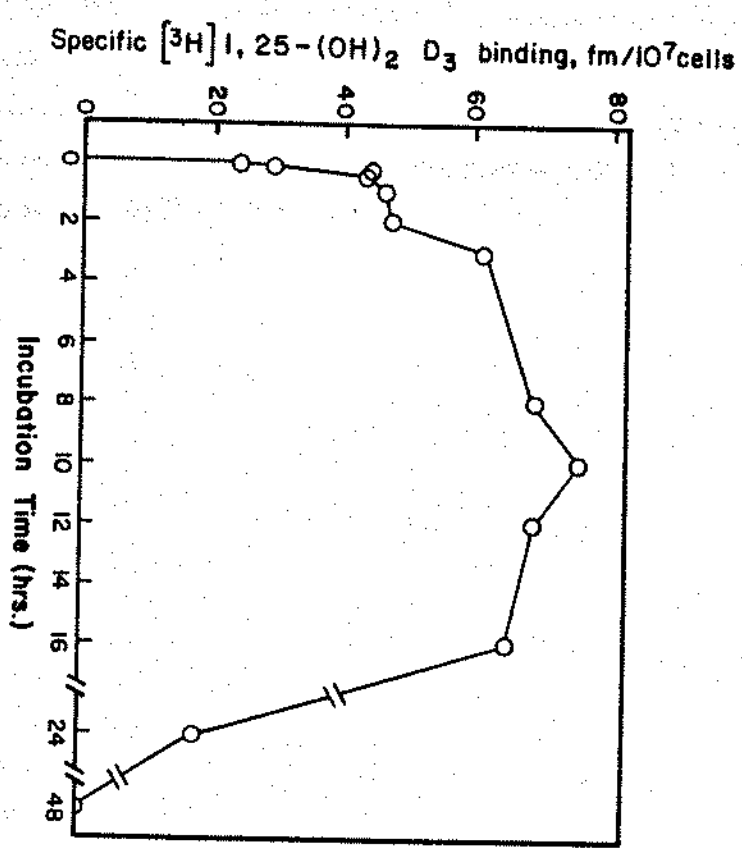

Specific [3H] 1, 25-(OH)2 D3 binding, fm/10^7cells
0
20
40
60
80
Incubation Time (hrs.)
0
2
4
6
8
10
12
14
16
24
48

Figure 2. Effect of addition of fresh hormone on loss of specific hormone binding on intact HL-60 cells. Cells (5×10^6) cultured in SFM were incubated with [^{3}H]1,25-$(OH)_2D_3$ as described in Fig. 1. Specifically bound [^{3}H]1,25-$(OH)_2D_3$ was quantitated by the HAP assay. The arrow indicates the second addition of fresh 1 nM [^{3}H]1,25-$(OH)_2D_3$ (●) at 24 h after the addition of first hormone. Values represent the mean for two replicate wells with duplicate measurements of each well.

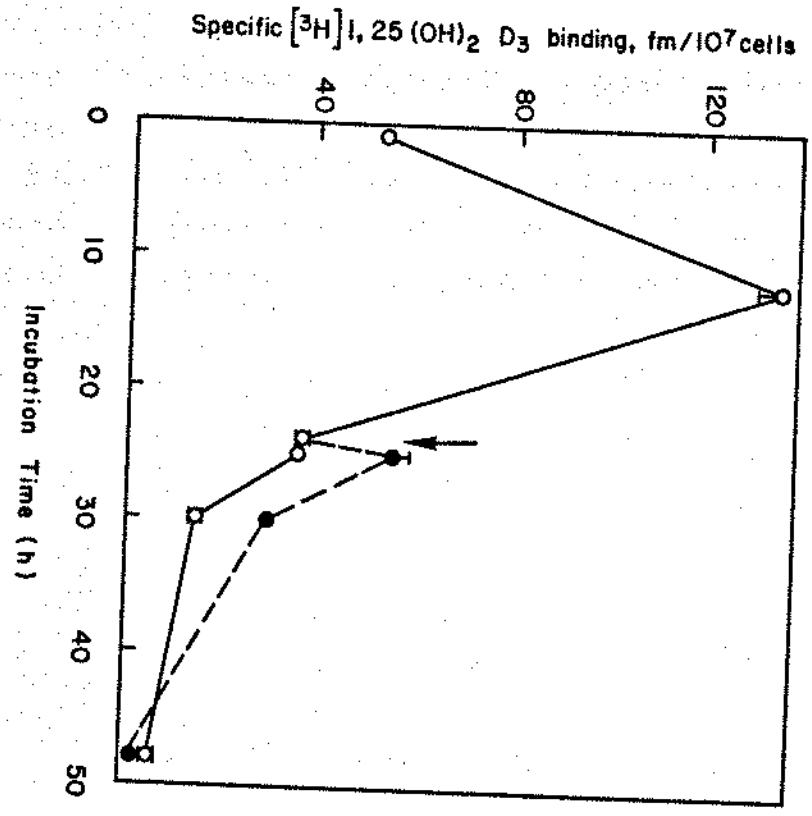

Specific [3H] I, 25 (OH)2 D3 binding, fm/10^7 cells
40
80
120
0
10
20
30
40
50
Incubation Time (h)

Figure 3. Time of displacement of [^{3}H]1,25-$(OH)_2D_3$ in nuclear receptor extracts by nonradioactive 1,25-$(OH)_2D_3$. HL-60 cells in SFM were incubated with 1.0 nM [^{3}H]1,25-$(OH)_2D_3$ for 2 h (Δ) or 12 h (O) and nuclear receptors extracted as described in the Materials and Methods. The extracts were further incubated with 5 mM diisopropyl fluorophosphate and 1.0 nM [^{3}H]1,25-$(OH)_2D_3$ in the presence (shaded symbols; nonspecific) or absence (open symbols; total) of a 200-fold molar excess of nonradioactive 1,25-$(OH)_2D_3$ at 30° C for up to 2 h. Nonspecific binding also was determined for cells incubated at 37° C in culture for 2 h (■) or 12 h (❑) in the presence of 1.0 nM [^{3}H]1,25-$(OH)_2D_3$ ± 200-fold excess of nonradioactive 1,25-$(OH)_2D_3$. Values are the mean of two separate experiments.

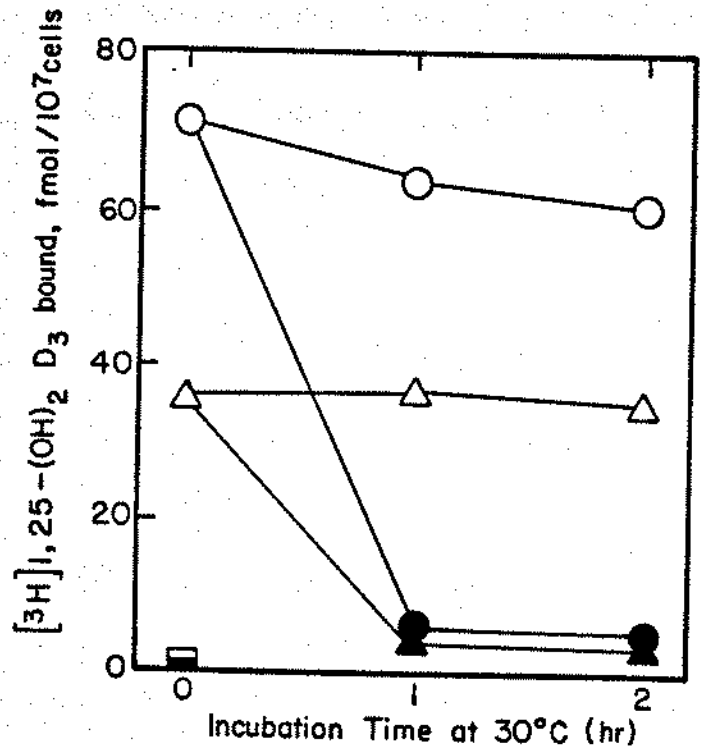

[3H]1,25-(OH)2 D3 bound, fmol/10^7cells
80
60
40
20
0
0
1
2
Incubation Time at 30°C (hr)

Figure 4. Effect of cell density on time-dependent regulation of 1,25-$(OH)_2D_3$ receptors in intact HL-60 cells. HL-60 cells were incubated at 37° C with saturating concentration of [^{3}H]1,25-$(OH)_2D_3$ (≥ 0.5 nM) in the presence or absence of 200-fold excess unlabeled hormone. Cells were seeded in SFM at the density of 5×10^5 (O), 2×10^6 (Δ), or 5×10^6 (●) cells/ml. The specific hormone binding activity was measured by HAP assay at the indicated times.

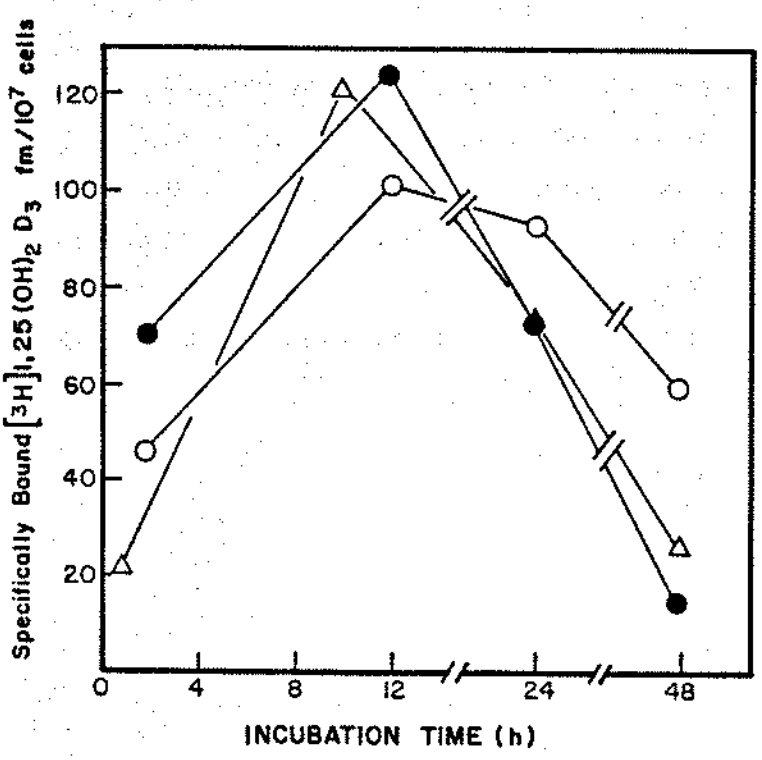

Specifically Bound [3H]1,25(OH)2 D3 fm/10^7 cells
120
100
80
60
40
20
0
4
8
12
24
48
INCUBATION TIME (h)

Figure 5. Determination of the apparent equilibrium dissociation constant (K_d) of 1,25-$(OH)_2D_3$ binding to its receptors in HL-60 cells. Cells (5×10^6) grown in SFM were labeled with increasing concentrations of [^{3}H]1,25-$(OH)_2D_3$ in the presence or absence of a 200-fold excess of nonradioactive 1,25-$(OH)_2D_3$ for 2 (A), 14 (B) and 24 h (C) at 37° C. Total binding (O), nonspecific binding (Δ), and specific binding radioactivity (●) were quantitated by the HAP batch assay. The data were analyzed by the method of Scatchard to determine K_d value from the slope of the plot with the x-intercept value (N_{max}) equaling the molarity of binding in solution (see inset for mean ± SE of replicate experiments). The K_d values for cells treated with 1,25-$(OH)_2D_3$ for 2, 14, and 24 h are not significantly different from each other as indicated by ANOVA ($P > 0.1$).

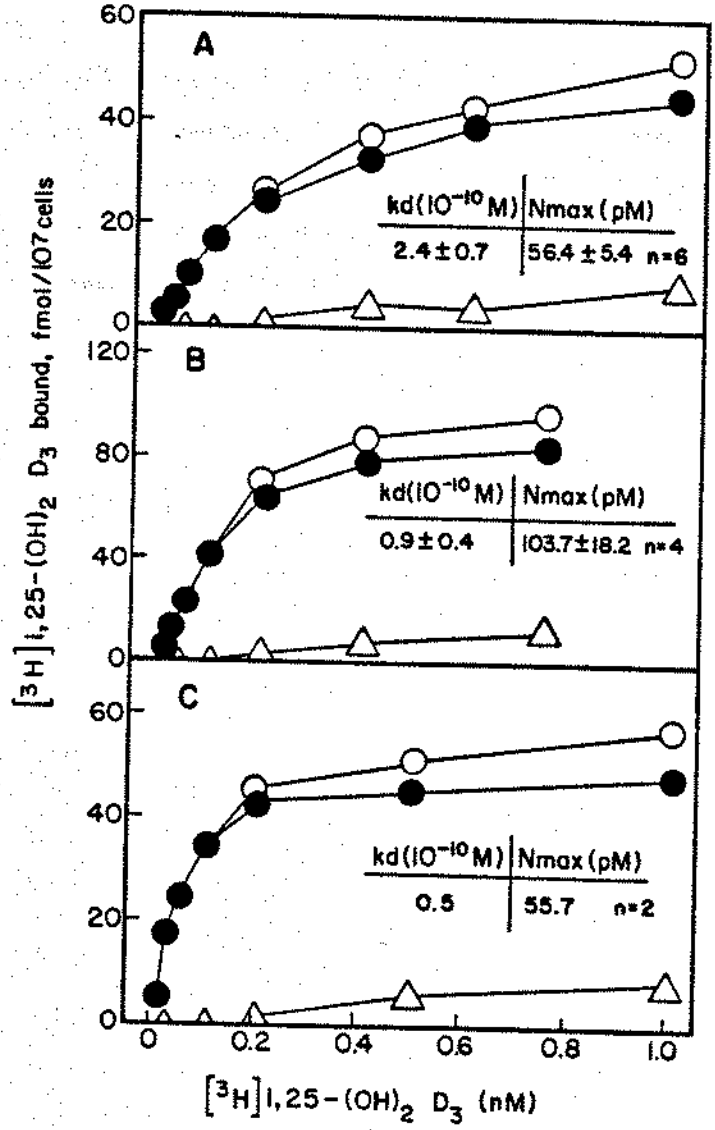

A
B
C
kd(10⁻¹⁰M) | Nmax (pM)
2.4±0.7 | 56.4±5.4 n=6
kd(10⁻¹⁰M) | Nmax (pM)
0.9±0.4 | 103.7±18.2 n=4
kd(10⁻¹⁰M) | Nmax (pM)
0.5 | 55.7 n=2
[³H]1,25-(OH)₂ D₃ bound, fmol/10⁷cells
[³H]1,25-(OH)₂ D₃ (nM)
0
20
40
60
80
120
0.2
0.4
0.6
0.8
1.0

Figure 6. Sucrose density gradient analysis of [^{3}H]1,25-$(OH)_2D_3$ labeled nuclear receptors in HL-60 cells grown in SFM. HL-60 cells (5×10^7) were treated with 1 nM [^{3}H]1,25-$(OH)_2D_3$ in the presence (●) or absence (O) of a 200-fold excess of unlabeled 1,25-$(OH)_2D_3$ for 2 (A), 12 (B), and 24 h (C) at 37° C in humidified 5% CO_2 atmosphere. Aliquots (0.25 ml) of [^{3}H]1,25-$(OH)_2D_3$ bound nuclear receptor were applied to linear sucrose density gradients (4-20%). Gradients were centrifuged for 18 h at 235,000 g and subsequently fractionated (0.1 ml). The arrow indicates the position of the marker protein, ovalbumin (3.7S).

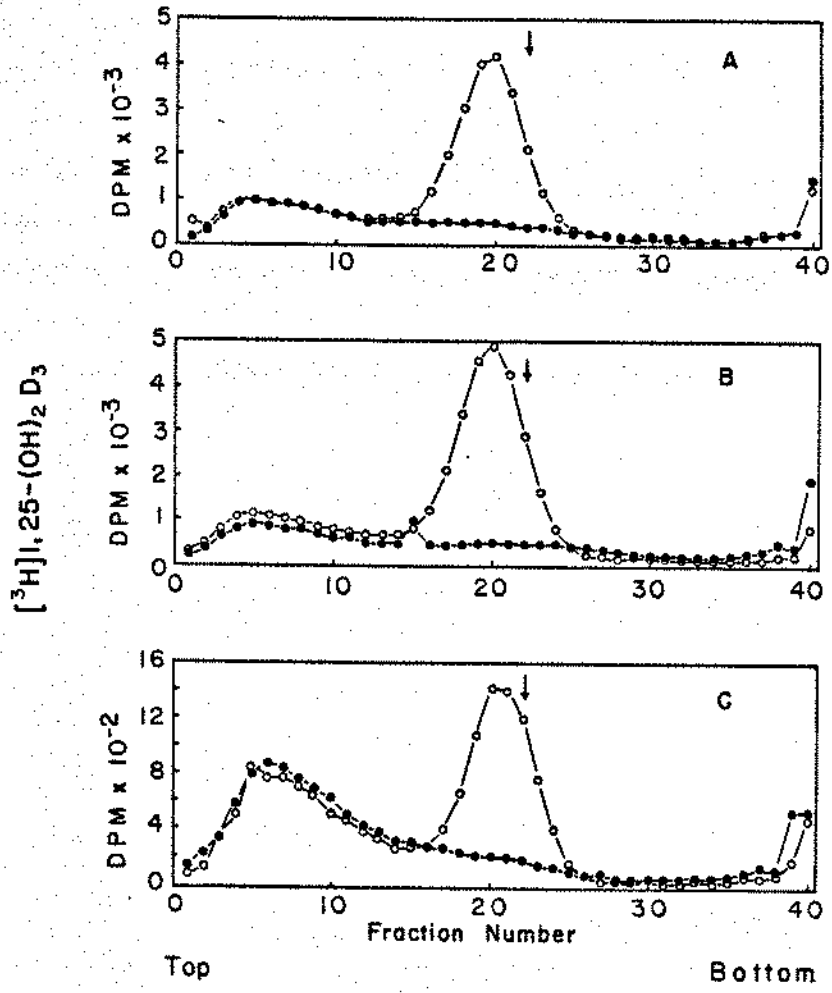

[3H]1,25-(OH)2 D3
DPM x 10-3
DPM x 10-2
A
B
C
Fraction Number
Top
Bottom

Figure 7. DNA-cellulose chromatography of [^{3}H]1,25-$(OH)_2D_3$ labeled nuclear receptor from intact HL-60 cells. Cells (5×10^7) grown in SFM were incubated with 1 nM [^{3}H]1,25-$(OH)_2D_3$ for 2 (A), 12 (B), and 24 h (C) at 37° C in humidified 5% CO_2 atmosphere. The [^{3}H]1,25-$(OH)_2D_3$ bound nuclear receptor, prepared as described in Materials and Methods section, was diluted ten times to reduce ionic strength (a final concentration of KCl equal to 0.03 M). Aliquots were applied to a 10 ml DNA-cellulose column. Chromatography was initiated by washing the column with TEDMK-0.03 M, followed by a linear KCl gradient (KCl 0.03-0.5 M). Arrow indicates the start of gradient. Fractions (2.5 ml) were collected and 1.5 ml-aliquots were removed to determine the radioactivity.

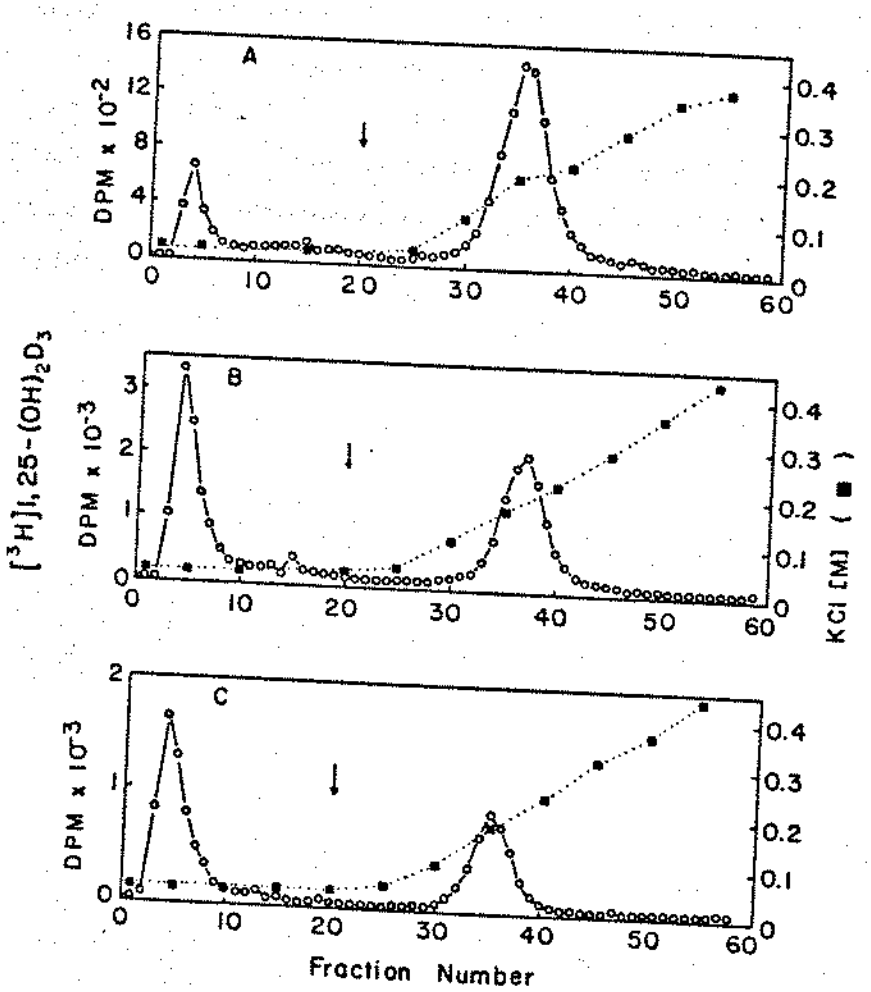

A
B
C
DPM x 10⁻²
DPM x 10⁻³
DPM x 10⁻³
[³H]1, 25-(OH)₂D₃
KCl [M] (■)
Fraction Number

Figure 8. Fast protein liquid chromatography (FPLC) of [^{3}H]1,25-$(OH)_2D_3$ labeled nuclear receptor from intact HL-60 cells cultured in SFM. Cells (5×10^7) were incubated with 1 nM [^{3}H]1,25-$(OH)_2D_3$ for 2 (A), 14 (B), and 24 h (C) at 37° C in a humidified 5% CO_2 atmosphere. Aliquots of tenfold diluted receptor, as described in Fig. 6, were injected onto an analytical strong anion exchange column (MonoQ®) (0.5 × 5 cm). Elution of receptor was carried out by employing a linear KCl gradient (0.03-0.4 M). Arrow indicates the start of gradient. Fractions (1.0 ml) were collected and 0.5 ml-aliquots were removed to determine the radioactivity.

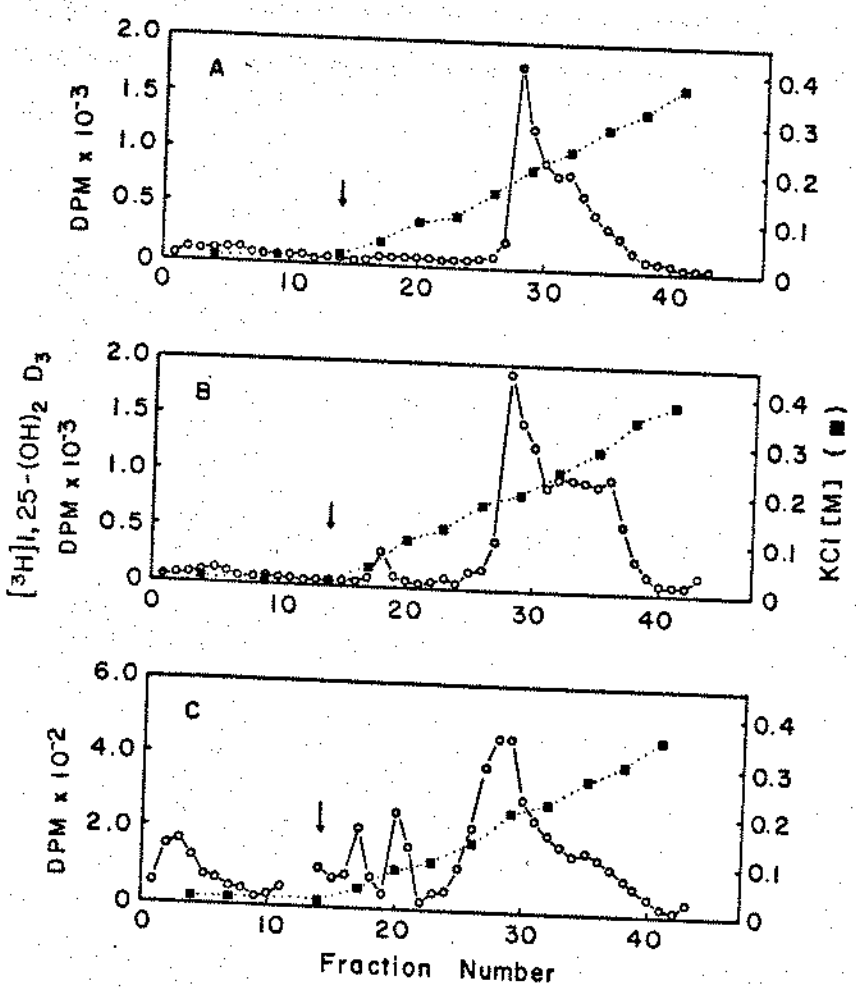

A
B
C
DPM x 10⁻³
DPM x 10⁻²
[³H]1, 25-(OH)₂ D₃
KCl [M] (■)
Fraction Number
0
10
20
30
40
0.5
1.0
1.5
2.0
4.0
6.0
0.1
0.2
0.3
0.4

Figure 9. Effect of hormone concentration on time-dependent specific 1,25-$(OH)_2D_3$ binding in intact HL-60 cells. The cells (5×10^6) in SFM were incubated with 0.05 nM (▲), 0.3 nM (O), 0.8 nM (●) and 1.5 nM (Δ) [^{3}H]1,25-$(OH)_2D_3$ in the presence or absence of a 200-fold molar excess of unlabeled hormone. Specific binding of hormone (fm/10^7 cells) was determined by HAP assay at the indicated times up to 24 h.

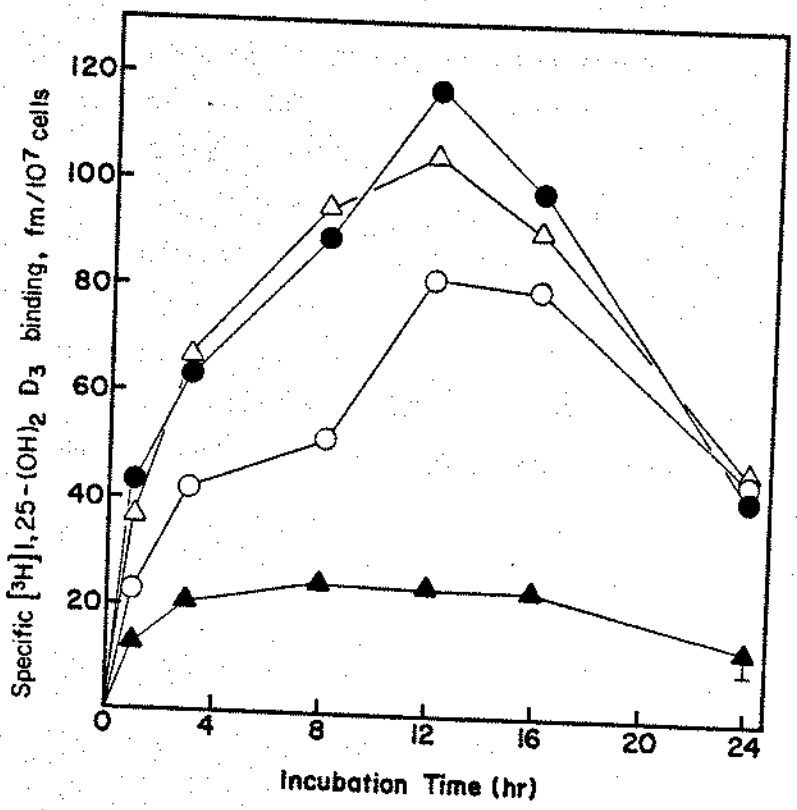

Specific [3H]1,25-(OH)2 D3 binding, fm/10^7 cells
120
100
80
60
40
20
0
4
8
12
16
20
24
Incubation Time (hr)

Figure 10. Quantitation of unoccupied and occupied 1,25-$(OH)_2D_3$ receptors for various doses of 1,25-$(OH)_2D_3$ during homologous up-regulation. HL-60 cells in SFM were incuated with various concentrations of [^{3}H]1,25-$(OH)_2D_3$ for 12 h at 37° C. Nonspecific binding was determined by incubating cells in the presence of a 200-fold molar excess of unlabeled hormone as described in the Methods. The treatment groups were divided and aliquots of cells were incubated with an additional dose of [^{3}H]1,25-$(OH)_2D_3$ 1 h prior to harvest (open bar), while the remaining aliquots received only vehicle (hatched bar). The basal level of receptors was measured by incubating cells with 1 nM [^{3}H]1,25-$(OH)_2D_3$ with or without 200-fold molar excess unlabeled hormone for 2 h at 37° C (solid bar). Specifically bound radioactivity was determined by HAP assay. Values are the mean for two separate experiments with duplicate wells being assayed in duplicate. The error bars represent the SE.

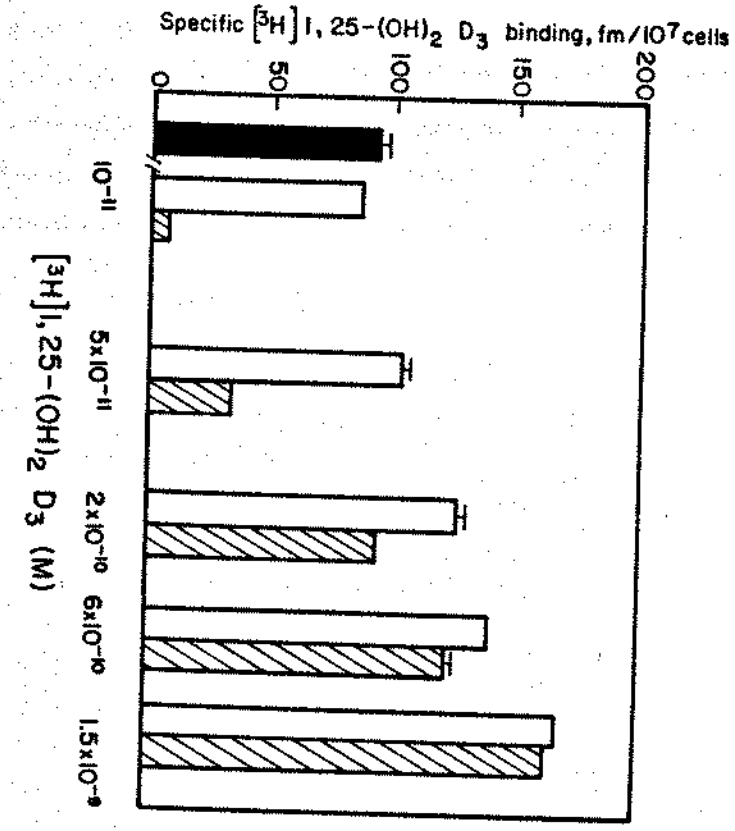

Specific [3H] 1, 25-(OH)2 D3 binding, fm/10^7 cells
0
50
100
150
200
10^-11
5x10^-11
2x10^-10
6x10^-10
1.5x10^-9
[3H]1, 25-(OH)2 D3 (M)

Figure 11. Effect of α-amanitin and actinomycin D on total mRNA synthesis in HL-60 cells measured by "Quick blot" assay. Cells (5×10^6) grown in SFM were treated with various concentrations of actinomycin D (A) or α-amanitin (B). A: Total mRNA synthesis was measured for cells treated with actinomycin D for 2 h. B: [^{3}H]uridine incorporation into mRNA was measured at 2 (O) or 10 h (Δ) after cells were subjected to electroporation in the presence of α-amanitin (see Materials and Methods section). Total mRNA synthesis was also measured for cells incubated with α-amanitin for 2 h without applying electroporation (●).

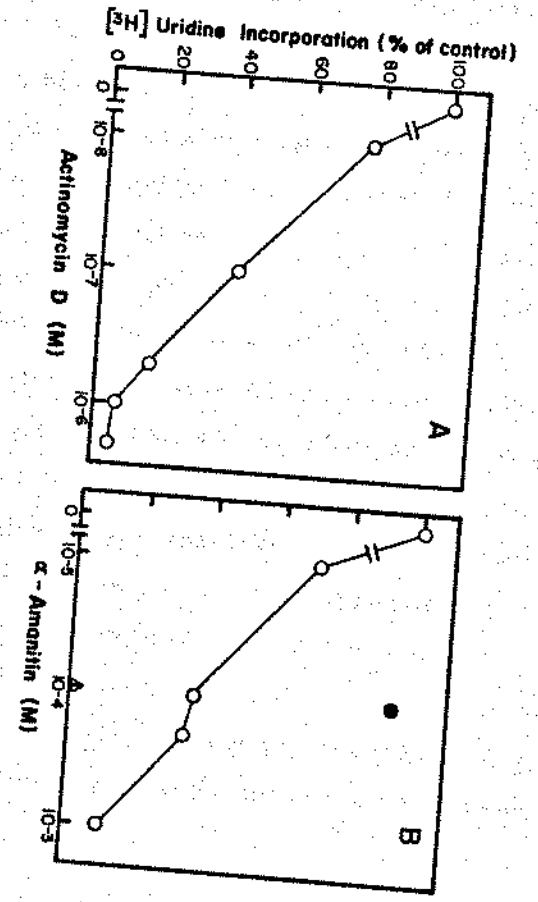

[3H] Uridine Incorporation (% of control)
0
20
40
60
80
100
Actinomycin D (M)
0
10-8
10-7
10-6
A
α - Amanitin (M)
0
10-5
10-4
10-3
B

Figure 12. Effect of inhibitors of transcription (A) and translation (B) on specific binding of 1,25-$(OH)_2D_3$. A: HL-60 cells were incubated with hormone (0.5 nM [^{3}H]1,25-$(OH)_2D_3$ ± 100 nM unlabeled hormone) alone (O) or in the presence of actinomycin D (2×10^{-6} M) (Δ), ethidium bromide (10^{-4} M) (▲). The cells pretreated with the same concentration of actinomycin D for 2, 6 or 12 h were labeled with radioactive hormone for 2 h (■). Electroporated cells were incubated with hormone alone (●) or in the presence of α-amanitin (2×10^{-4}) (❑). Data in B show the effect of cycloheximide (10^{-5} M) on the apparent up- (Δ) and down-regulation (●) of 1,25-$(OH)_2D_3$ receptor. Cells were incubated with hormone (0.5 nM [^{3}H]1,25-$(OH)_2D_3$ ± 200 nM unlabeled hormone) alone (O) or in the presence of cycloheximide (Δ). In separate experiments, cycloheximide was added to medium at 12 h after the addition of hormone to cells (●). The cells pretreated with cycloheximide (10^{-5} M) for 2, 12 or 24 h were labeled with radioactive hormone for 2 h (▲). Specifically bound radioactivity was determined at times indicated by HAP assay. Data is expressed as percent of basal receptor level from HL-60 cells incubated with hormone for 2 h. The basal receptor level was an average of 29.3 ± 0.7 fm and 40.5 ± 2.8 fm per 10^7 cells for electroporated and control cells, respectively in (A) and 53.8 ± 6.6 fm per 10^7 cells in (B). Values represent the mean ± SE for an average of four replicate wells with duplicate measurement of each well.

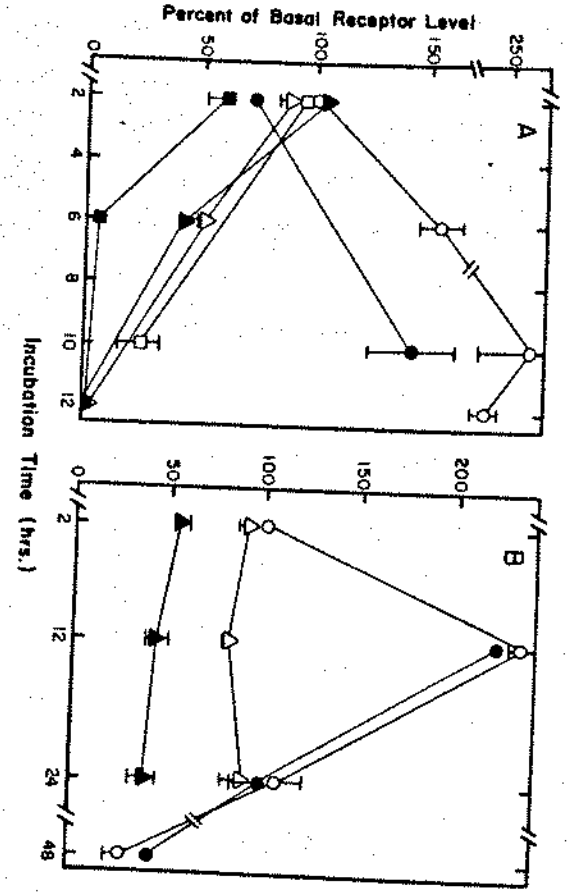
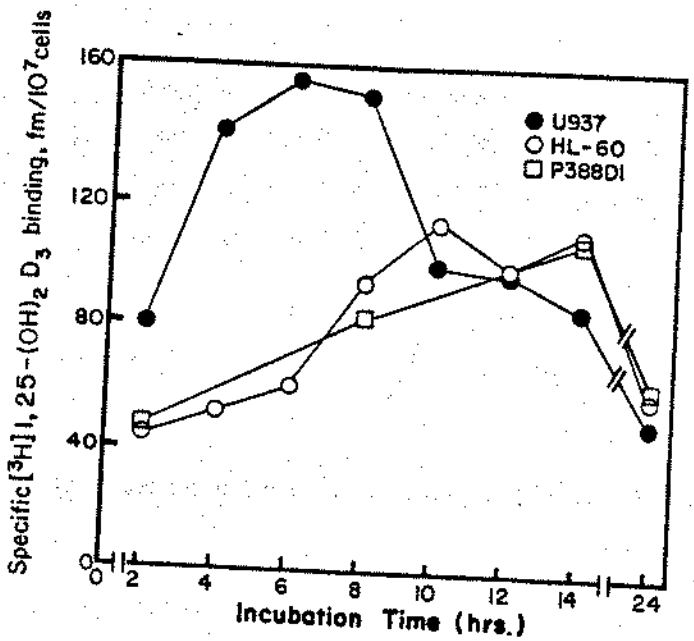

Percent of Basal Receptor Level
0
50
100
150
250
A
2
4
6
8
10
12
Incubation Time (hrs.)
0
50
100
150
200
B
2
12
24
48

Figure 13. Time-dependent regulation of [^{3}H]1,25-$(OH)_2D_3$ uptake into intact U937 and P388D1 cells in comparison to that of HL-60 cells. HL-60 cells (5×10^6) (O) grown in SFM were incubated in SFM containing 0.5 nM [^{3}H]1,25-$(OH)_2D_3$ in the presence or absence of a 200-fold excess of unlabeled 1,25-$(OH)_2D_3$ at 37° C in humidified 5% CO_2 atmosphere. U937 (●) and P388D1 (❑) (5×10^6 cells) cultured in 10% SCM were incubated in 1% SCM with the same concentration of hormone. Specifically bound radioactivity was determined by HAP assay at times indicated. Values represent the mean for average of three replicate experiments with duplicate measurement.


Specific [3H]1, 25-(OH)2 D3 binding, fm/10^7cells
160
120
80
40
0
0
2
4
6
8
10
12
14
24
Incubation Time (hrs.)
U937
HL-60
P388D1

CHAPTER IV

IMMUNOLOGICAL IDENTIFICATION OF 1,25-DIHYDROXYVITAMIN D_3 RECEPTORS IN HUMAN PROMYELOCYTIC LEUKEMIC CELLS (HL-60) DURING HOMOLOGOUS REGULATION

Youngsook Lee, Masaki Inaba, Hector F. DeLuca and William S. Mellon

(Submitted to *Journal of Biological Chemistry*, 1988)

SUMMARY

We have reported previously that the receptors for 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) are up- and down-regulated homologously in intact promyelocytic leukemic cells, HL-60, upon continuous exposure to 1,25-$(OH)_2D_3$. These findings were based on receptor quantification by tritiated ligand binding studies. Here, immunologic techniques were utilized to detect receptor levels and further characterize physical/chemical changes in the receptor during the homologous regulation. The monoclonal antibody (IVG8C11) raised against the porcine intestinal 1,25-$(OH)_2D_3$ receptor immunoprecipitated quantitatively 1,25-$(OH)_2D_3$ receptors in the nuclear extracts from HL-60 cells. The highly enriched immunoprecipitated receptors were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were then probed with ^{125}I-IVG8C11 and autoradiographed at -70° C. The basal receptor from the cells treated with 1,25-$(OH)_2D_3$ for 2 h was detected as a single form at 53 kDa. The putative up-and down-regulated receptors from the cells treated with 1,25-$(OH)_2D_3$ for 12, 48, and 72 h, respectively, were also detected as a single form and have the same molecular weight as the basal receptor. The ratios of densities of the bands were 1.0:4.2:1.2:0.9 for receptors from the cells treated with 1,25-$(OH)_2D_3$ for 2, 12, 48, and 72 h, respectively, as measured by Soft Laser scanning densitometer. These data, together with parallel binding studies, clearly indicate the induction of 1,25-$(OH)_2D_3$ receptor by its ligand results from actual increases in receptor protein and a loss of hormone binding activity from a decrease in receptor protein and further by a loss of 1,25-$(OH)_2D_3$ binding activity of receptors. The up-regulation of receptors was completely inhibited by cycloheximide, 10 μM, as indicated by the

[^{3}H]1,25-$(OH)_2D_3$ binding studies as well as immunoblotting experiments. In order to examine the specificity of the receptor detection, the monoclonal antibody, XVIE6E6G10, which recognizes only the pig receptors for 1,25-$(OH)_2D_3$, was used to immunoprecipitate the receptors from HL-60 and pig intestine followed by immunoblotting with ^{125}I-IVG8C11. The 1,25-$(OH)_2D_3$ receptor in HL-60 cells was not detected, while the pig intestinal receptor was detected at 55 kDa as a single protein species, indicating the specificity of the receptor detection system. Therefore, the data presented here strongly support the hypothesis of homologous regulation of 1,25-$(OH)_2D_3$ receptors in intact human target cells.

INTRODUCTION

The actions of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) in target cells are believed to be mediated by the interaction of the hormone with a specific intracellular receptor protein (1,2). The receptor-hormone complex is postulated to regulate the expression of specific hormone-dependent genes, possibly by binding to promoter regions of genes, in a manner similar to that hypothesized for other steroid hormones (3,4). Receptors for 1,25-$(OH)_2D_3$ have been recently identified in hematopoietic cells including normal human lymphocytes and monocytes (5,6) as well as malignant cell lines with monocytic and myeloid characteristics (7-10). It has been shown that 1,25-$(OH)_2D_3$ promotes *in vitro* differentiation of normal bone marrow cells, peripheral human monocytes, and regulates directly macrophage function (11-13). Moreover monoblastic and myeloblastic cell lines (14-16), including the human promyelocytic leukemic cell line, HL-60 cell (8,11) are differentiated into macrophages in the presence of 1,25-$(OH)_2D_3$.

The possibility that 1,25-$(OH)_2D_3$ may be involved in the maturational processing of hematolymphopoietic cells through the receptor mediated mechanism has been suggested. The existence of a positive correlation between 1,25-$(OH)_2D_3$-induced differentiation and the occurrence of occupied 1,25-$(OH)_2D_3$ receptors has been reported in HL-60 cells (8). The functional defect of 1,25-$(OH)_2D_3$ resistant HL-60 clones has been correlated with a reduced amount of specific 1,25-$(OH)_2D_3$ receptors (17). The rank order of vitamin D_3 metabolites inducing monocytic differentiation is similar to their affinity to the 1,25-$(OH)_2D_3$ receptor in HL-60 cells (7,18).

Receptor presence for 1,25-$(OH)_2D_3$ sensitive target cells has been correlated also with the magnitude of the biological response in cultured cell lines

including several rat osteogenic sarcoma cell lines, a kidney and a mouse osteoblast-like cell line (19-21). Therefore, alteration of receptor level is an important factor in modulating cell responsiveness to hormone. Both homologous and heterologous regulation of 1,25-$(OH)_2D_3$ receptor have been reported *in vivo* and *in vitro* by using radiolabeled hormone-receptor binding techniques. The level of 1,25-$(OH)_2D_3$ receptors undergoes homologous up-regulation by 1,25-$(OH)_2D_3$ in a cultured kidney cell line and in the rat kidney *in vivo* (22,23). The homologous down-regulation of 1,25-$(OH)_2D_3$ receptors has been reported in intact human breast cancer cells and ligand-dependent regulation of the 1,25-$(OH)_2D_3$ receptors in rat osteosarcoma cells (24,25).

However, relatively little is known about mammalian 1,25-$(OH)_2D_3$ receptor partly due to the low abundance of 1,25-$(OH)_2D_3$ receptor ($\leq$ 0.001% of total cytosolic protein) and/or extreme proteolytic sensitivity during isolation. Moreover, the information on the biosynthesis of 1,25-$(OH)_2D_3$ receptor and its regulation by 1,25-$(OH)_2D_3$ are limited. Because monoclonal antibodies can be generated to specific antigenic sites and potentially different functional domains on the protein, they should serve as useful tools to examine protein structure and function. The hybridoma technique has been applied to the study of many different steroid hormone receptors including estrogen (26-28), progesterone (29-31), glucocorticoid (32-35) as well as 1,25-$(OH)_2D_3$ in chicken (36,37). Monoclonal antibodies to the porcine 1,25-$(OH)_2D_3$ receptor have been produced (38) and employed for the characterization of pig 1,25-$(OH)_2D_3$ receptor as a 55-kDa protein with the major form having an isoelectric point of 6.1 (39,40).

We have previously reported the level of 1,25-$(OH)_2D_3$ receptors in intact HL-60 cells increased (up-regulation) and then decreased (down-regulation) upon

continuous exposure to 1,25-$(OH)_2D_3$ (41). Since these findings were based on receptor quantification by high specific activity tritiated ligand, potentially non-hormone binding or unoccupied forms of the receptor cannot be characterized from this studies. In the present studies, we have attempted to further characterize and detect any changes that occur in the 1,25-$(OH)_2D_3$ receptor during homologous regulation using immunological techniques. Immunological detection of 1,25-$(OH)_2D_3$ receptor was performed by immunoprecipitation, resolution by SDS-polyacrylamide gel electrophoresis and immunoblotting using monoclonal antibody raised against pig 1,25-$(OH)_2D_3$ receptor with high cross reactivity with mammalian receptors (38). In this paper, we provide strong evidence that up-and down-regulated receptors have the same electrophoretic mobility as that of basal receptors and these three receptor groups exist as one antigenically related species of 53,000 molecular weight. These data also support the hypothesis of receptor autoregulation in that the presence of 1,25-$(OH)_2D_3$ leads to a significant increase followed by a decrease in the amount of 1,25-$(OH)_2D_3$ receptor in HL-60 cells.

MATERIALS AND METHODS

Materials. 1α,25-dihydroxyvitamin D_3, radiolabeled with tritium at the 26,27-methyl groups ([^{3}H] 1,25-$(OH)_2D_3$, 160 Ci/mmol), was obtained from Du Pont, NEN Research Products (Boston, MA). Nonradioactive 1,25-$(OH)_2D_3$ was a gift from Dr. M. Uskokovic of Hoffmann-LaRoche Inc., (Nutley, NJ). Determinations of purity and concentration of 1,25-$(OH)_2D_3$ were achieved by UV absorption spectroscopy using an extinction coefficient (E = 264) of 18,200 M^{-1} cm^{-1}. Hydroxylapatite, acrylamide and bis-acrylamide were purchased from BIO-RAD (Richmond, CA), Sephadex G-25 (med), particle size 50-150 μm, from

Pharmacia Inc., (Piscataway, NJ), glycine and sodium dodecyl sulfate (SDS) from Schwarz/Mann Biotech (Division of ICN Biomedicals, Inc., Cleveland, OH). Prestained protein molecular weight standards (molecular weight ranges from 14,300-200,000) were obtained from Bethesda Research Laboratories Life Technologies, Inc., (Gaithersburg, MD).

Cell Culture. The human promyelocytic leukemic cells, HL-60, were maintained in RPMI-1640 medium buffered with 25 mM Hepes (Sigma Chemical Co., St. Louis, MO) containing 100 units/ml penicillin and 0.5 μg/ml streptomycin. This basal medium was supplemented with a defined supplement of insulin from bovine pancreas (5 μg/ml), human transferrin (5 μg/ml) , sodium selenite (5 ng/ml), and BSA (fatty acid free, 0.5 mg/ml) (Sigma Chemical Co., St. Louis, MO), designated as a serum free medium, SFM (42). HL-60 cells were grown in SFM at 37° C in a humidified 95% air-5% CO_2 atmosphere and subcultured with 4 day intervals at a seeding density of 3×10^5 cells per ml. Cell proliferation was assessed by counting cells in a hemocytometer and cell viability was determined by trypan blue dye exclusion.

Preparation of 1,25-$(OH)_2D_3$ Receptors. Receptors for 1,25-$(OH)_2D_3$ from HL-60 cells were prepared as described previously (42). Briefly, HL-60 cells cultured in SFM were incubated with 2 nM 1,25-$(OH)_2D_3$ in SFM at 37° C in humidified 95% air-5% CO_2 atmosphere for various times. The following procedures were done at 4° C. After cells were washed twice in PBS, they were resuspended and incubated for 20 min in TEDM buffer with intermittent vortexing followed by homogenization using a Tissuemizer type SDT (Tekmar Co., Cincinnati, OH) for 3 sec at a speed setting of 50. The homogenate was

centrifuged for 10 min at 800 g to obtain crude nuclear fractions. The crude nuclear pellet was incubated in TEDMK-0.3 for 30 min followed by centrifugation at 105,000 g for 45 min in a Beckman L5-50B ultracentrifuge using a type Ti-50 rotor (Beckman Instruments, Palo Alto, CA). The supernatant was utilized as the source of occupied calcitriol receptors in HL-60 cells for electrophoresis experiments. The nuclear extracts from porcine intestine were prepared as described previously (39). The total cellular extracts of HL-60 cells were prepared essentially as described previously (43). All operations were performed at 4° C. After cells were washed 3 times with PBS, they were suspended in TEDK-0.3 with a protease inhibitor, 5 mM diisopropyl phosphorofluoridate (DFP) and sonicated with three 20-sec cycles interrupted by 60-sec pauses. The sonicate was centrifuged at 105,000 g for 60 min to yield the total cellular receptor preparation.

Measurement of Total Cellular Receptors. The amount of total cellular receptors for 1,25-$(OH)_2D_3$ was determined in HL-60 cells as described previously (42). Briefly, cells in SFM were incubated with various concentrations of [^{3}H]1,25-$(OH)_2D_3$ in the presence or absence of a 200-fold excess of nonradioactive hormone. After the each incubation period, cells were washed twice with PBS and resuspended in TEDM buffer for 20 min at 4° C with intermittent vortexing. Aliquots of cells in TEDM buffer were assayed for receptor-bound [^{3}H]1,25-$(OH)_2D_3$ by hydroxylapatite (HAP) assay (44) with slight modification (42). Specific binding was estimated as the difference between total binding ([^{3}H]1,25-$(OH)_2D_3$ alone) and nonspecific binding (with a 200-fold excess nonradiolabeled hormone). The radioactivity was measured by liquid scintillation spectrometry (Packard Tri-Carb Spectrometer, model Prias PLD) using Opti-fluor

Scintillation fluid (Packard, Downers Grove, IL) with an efficiency for tritium of 30%.

Immunóprecipitation. The monoclonal antibodies to the porcine intestinal 1,25-$(OH)_2D_3$ receptor used in this work, IVG8C11 (IVG) and XVIE6E6G10 (XVI), were described previously (38). The nuclear extracts from HL-60 cells treated with 1,25-$(OH)_2D_3$ or [^{3}H]1,25-$(OH)_2D_3$ were incubated with 5-10 ng of monoclonal antibodies to immunoprecipitate 1 fm of 1,25-$(OH)_2D_3$ receptor with continuous shaking for 16 h at 4° C. The pig nuclear extract was incubated simultaneously with [^{3}H]1,25-$(OH)_2D_3$ in the presence or absence of a 200-fold excess of nonradiolabeled hormone and with monoclonal antibodies. For precipitation, 50 µl of goat anti-mouse IgG-Sepharose (Organon Teknika Corporation, West Chester, PA) in a 50% slurry in PBS was added to each tube and incubated for 1 h at 4° C with continuous shaking. Following incubation, the tubes were centrifuged at 1,500 g for 5 min, the supernatant removed for binding assay or discarded, and the Sepharose pellets washed twice by resuspension with 1 ml of PBS containing 0.5% triton x100 followed by centrifugation. To determine the efficiency of immunoprecipitation, the Sepharose beads incubated with [^{3}H]1,25-$(OH)_2D_3$ labeled receptor were transferred to scintillation vials with two 200-µl washes of ethanol and 4 ml of Opti-fluor scintillation fluid added. The radioactivity was measured as described above. To electrophorese the immunoprecipitated receptors, the Sepharose beads incubated with occupied 1,25-$(OH)_2D_3$ receptors were boiled for 5 min in 1 vol of SDS-denaturing buffer. After the tubes were centrifuged at 200 g for 10 min at 25° C, the supernatants were electrophoresed on 9% SDS-polyacrylamide gels (1.5 mm spacer) as described by Laemmli (45).

Immunoblot. For use in immunoblotting experiments, purified monoclonal antibody, IVG, was iodinated with ^{125}I-Bolton-Hunter reagent (NEN Research Products) as described previously (46). Briefly, 10 μg of IVG in 10 μl of PBS were reacted with ^{125}I-Bolton-Hunter reagent (1 mci) for 2 h on ice. To stop the reaction, 0.25 ml of glycine in 0.1M borate (pH 8.5) was added for 5 min, then 0.25 ml of PBS containing 0.25%gelatin was added to the reaction vial. The monoclonal antibody coupled with ^{125}I was separated from free ^{125}I using Sephadex G-25 (medium) column, which was loaded with 1 ml of 3% (w/v) bovine serum albumin in PBS and then equilibrated with PBS containing 0.25% gelatin. Fractions were collected (0.5 ml) and 5 μl-aliquots were removed from each fraction to determine the radioactivity in a Prias PLD scintillation spectrometer (Packard) using a channel optimized for determination of ^{125}I. After the peak fractions were combined, 10 μl-aliquots were removed to measure radioactivity. ^{125}I-IVG was examined using deoxycholate-TCA precipitation method (80% of the radioactivity was precipitated by incubation with 100 μl of 0.15% deoxycholate and 100 μl of 72% TCA.) After electrophoresis, the gels were equilibrated in transfer buffer for 15 min and transferred electrophoretically to polyvinylidene difluoride (PVDF) membrane (Milipore Corporation, Bedford, MA) at 145 mA for 16 h using a Transphor electrophoresis unit (Hoefer Scientific Instrument, San Francisco, CA). The PVDF membrane was prewet in 100% methanol for 1-2 sec, rinsed in water for 5 min and then equilibrated with transfer buffer for 10-15 min prior to use in blotting. After electrophoretic transfer, the membranes were washed with PBS for 10 min to remove transfer buffer, blocked with 5% blotto for 2 h at room temperature, and rinsed with 0.5% blotto for 2-5 min. The membranes were then incubated with 3×10^5 cpm/ml of ^{125}I-IVG in 0.5% blotto for 3 h. After the

membranes were washed five times for 10 min each with 0.5% blotto and rinsed in PBS, they were air-dried, and autoradiographed using Kodak XAR2 film with Du Pont Cronex Quanta III enhancing screens (Du Pont, New England Nuclear) at -70° C.

Buffers. The compositions of buffers used in these experiments are as follows: Phosphate-buffered saline (PBS), 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 8.0, 137 mM NaCl, 2.7 mM KCl; Tris-buffered saline with Tween 20 (TBST), 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20; Blocking buffer (Blotto), 5% (w/v) Carnation non-fat dry milk and 0.02% (W/V) NaN_3 in TBST; SDS-denaturing buffer, 0.125 M Tris, pH 6.8, 4% SDS (w/v), 20% (v/v) glycerol, 0.002% Bromophenol blue, 10% mercaptoethanol ; Gel running buffer, 0.025 M Tris, pH 8.3, 0.192 M glycine, 0.1% SDS; transfer buffer, 0.025 M Tris, pH 8.3, 0.192 M glycine, 0.1% (w/v) SDS, 20% (v/v) methanol; TEDM, 50 mM Tris-HCl, pH 7.5, 1.5 mM EDTA, 5 mM dithiothreitol, 10 mM sodium molybdate; TEDMK-0.3, TEDM buffer with 0.3 M KCl

RESULTS

To characterize the 1,25-$(OH)_2D_3$ receptors in the HL-60 cells during homologous regulation, monoclonal antibodies to the 1,25-$(OH)_2D_3$ receptor was used in immunoprecipitation and immunoblotting. The monoclonal antibody, IVG raised against the porcine intestinal receptor for 1,25-$(OH)_2D_3$ were used for immunoprecipitation to concentrate 1,25-$(OH)_2D_3$ receptor in HL-60 cells. To assess the ability of monoclonal antibody to bind the receptor in the crude nuclear fraction of HL-60, the immunoprecipitation efficiency for HL-60 receptor was

determined and compared to that of porcine intestinal receptor (Fig.1). To a constant amount of pig or HL-60 cell nuclear extracts, increasing amounts of IVG were used to immunoprecipitate receptors. To precipitate the antibody-receptor complex, goat anti-mouse IgG coupled to Sepharose were added. All of HL-60 cell receptors, occupied *in vitro*, were immunoprecipitated quantitatively by IVG in a similar manner to the immunoprecipitation efficiency of the pig intestinal receptor. These titration experiments showed that 10 ng of IVG was sufficient to precipitate 1 fm of [^{3}H]1,25-$(OH)_2D_3$ bound receptors. This ratio was used for all immunoprecipitation for immunoblotting experiments. The hormone binding activity was determined by HAP assay as described in the Materials and Methods.

Immunoblotting experiments were performed to characterize the basal, apparent up- and down-regulated receptor for 1,25-$(OH)_2D_3$ in HL-60 cells. HL-60 cells were incubated with 2 nM 1,25-$(OH)_2D_3$ for 2, 12, and 36 h to prepare the basal, up,and down-regulated 1,25-$(OH)_2D_3$ receptor, respectively. The 1,25-$(OH)_2D_3$ receptors in nuclear extracts were immunoprecipitated and then electrophoresed on 9% discontinuous SDS-polyacrylamide gels. After the proteins in the gels were transferred to PVDF membrane, 1,25-$(OH)_2D_3$ receptors were detected by probing the membrane with ^{125}I-IVG. As shown in Fig.2, the basal receptor, occupied *in vivo*, was detected as a single form of 53,000 molecular weight (lane 1). The up-regulated (lane 3) and down-regulated (lane 5) receptors were detected as a single form of 53,000. The ratios of densities for three bands were 1.0:3.2:1.2 for basal, up and down-regulated receptor, respectively, as measured by Soft Laser scanning densitometer, model SL-504-XL (Biomed Instruments, INC., Fullerton, CA). These data indicated clearly that there was a 3-fold increase in the amount of protein detected by IVG in lane 3 compared to the

basal receptor level, lane 1. This phase was followed by the decrease in the level of receptor to the basal receptor level by incubating cells with 1,25-$(OH)_2D_3$ for 36 h (lane 5). The increase in the amount of receptor during up-regulation was blocked completely in the presence of 10 μM cycloheximide (lane 2). The cell viability was greater than 90% throughout the experiments as examined by trypan blue exclusion. To determine the immunoprecipitation efficiency, parallel flasks of cells were incubated with 2 nM [^{3}H] 1,25-$(OH)_2D_3$. The immunoprecipitation efficiencies ranged from 85 to 90%.

The regulation of [^{3}H]1,25-$(OH)_2D_3$ uptake into intact cells in the presence or absence of cycloheximide is shown in Fig. 3. Receptors for 1,25-$(OH)_2D_3$ became saturated within 1 h upon incubation of HL-60 cells with concentrations of [^{3}H]1,25-$(OH)_2D_3$ greater than 0.8 nM as previously demonstrated (42). The amount of specific binding increased and became maximal at about 12 h. This up-regulated phase was followed by apparent down-regulation observed at 24 h. Up regulation was prevented in HL-60 cells incubated in the presence of [^{3}H]1,25-$(OH)_2D_3$ and the protein synthesis inhibitor, cycloheximide. Moreover, receptors were barely detectable by either immunodetection (Fig. 2) or [^{3}H]1,25-$(OH)_2D_3$ binding at 36 h in the cycloheximide treated cells. This concentration of cycloheximide inhibited protein synthesis by 92% as measured by [^{3}H]leucine incorporation and had no deleterious effect in cell viability (data not shown).

In order to examine whether the level of receptor decreased to below that of basal level upon prolonged incubation, an immunoblot assay was performed for the cells treated with hormone for up to 72 h (Fig. 4). Cells were seeded at a lower density, 5×10^5 cells/ml, to exclude the possibility of the lack of nutrients in the medium with the extended incubation periods. Fresh hormone was added to the

cells with 48 and 72 h incubation 24 h prior to the harvest. The level of immunoprecipitated receptor reached maximum between 12-24 h followed by decreases to the basal receptor level in 48-72 h. The ratios of densities for the bands were 1.0: 4.2: 3.9: 1.2: 0.9 for 2, 12, 24, 48 and 72 h treatment with hormone, respectively. The cells were labeled with [^{3}H]1,25-$(OH)_2D_3$ in the presence or absence of 200-fold excess nonradioactive hormone in the parallel flasks to measure the specific hormone binding activities in cells (Fig. 5). The specific hormone binding activities were maximal between 12-24 h followed by a decrease in the binding.

The monoclonal antibody, XVI, characterized by its specificity to only pig receptor (38) was examined for its immunoprecipitation efficiency for *in vivo* occupied receptor from HL-60. As shown Fig.6, IVG precipitated 97% and 99% of the receptor in the HL-60 cells and pig nuclear extract, respectively, while XVI did not immunoprecipitate the receptor in HL-60 cell nuclear extract. These results indicate clearly that all of the [^{3}H]1,25-$(OH)_2D_3$ binding activities in nuclear extract from HL-60 cells labeled *in vivo* are recognized by IVG but not by XVI. To examine the specificity of the immunoprecipitation and immunoblot assay for the detection of receptors in HL-60 cells, the monoclonal antibody XVI was used to immunoprecipitate the receptors in nuclear extracts of porcine intestine or HL-60 cells (Fig.7). The pig nuclear receptors immunoprecipitated by XVI (lane 2) have the same electrophoretic mobilities as the receptor immunoprecipitated by IVG (lane 1) and both were detected as a single band at 55 kDa. The pig nuclear receptors loaded on the gels without the immunoprecipitation procedure (lane 3) were detected at the same position as receptors that were immunoprecipitated. However, the immunoprecipitated receptor in HL-60 cells by XVI was not observed by

immunoblotting (lane 5), while the immunoprecipitated receptor by IVG was detected at 53 kDa.

DISCUSSION

In the present study, we utilized immunological techniques to identify and detect any changes occurring during the homologous regulation of 1,25-$(OH)_2D_3$ receptor in HL-60 cells. The results presented here provide strong evidence that 1,25-$(OH)_2D_3$ autoregulates its receptor level in intact HL-60 cells in a time-dependent manner. This approach represents an extension of earlier work in which 1,25-$(OH)_2D_3$ may exert a complex regulatory control of its receptor level (41). Previous observations have shown that the receptors for 1,25-$(OH)_2D_3$ in HL-60 cells were saturated with [^{3}H]1,25-$(OH)_2D_3$ in about 1 h (basal level) followed by 2-3 fold increases in specific [^{3}H]1,25-$(OH)_2D_3$ binding (apparent "up-regulation") by 12 h exposure to 1,25-$(OH)_2D_3$. This maximal binding activity was followed by the decrease in [^{3}H]1,25-$(OH)_2D_3$ binding activity (apparent "down-regulation") observed by 24-48 h during continuous 1,25-$(OH)_2D_3$ exposure. In addition, the homologous regulation of 1,25-$(OH)_2D_3$ receptors has been reported in several cultured cell lines and tissues using [^{3}H]1,25-$(OH)_2D_3$ binding studies (22-25).

However, conclusions regarding the location and/or quantification of unoccupied or potentially non-hormone binding forms of the receptor cannot be drawn from these ligand binding studies. The availability of monoclonal antibodies to the 1,25-$(OH)_2D_3$ receptor enables the study of this protein independent of hormone-binding activity. The monoclonal antibodies used in this study were raised against the porcine intestinal 1,25-$(OH)_2D_3$ receptor, as described previously (38). The monoclonal antibody, IVG, is characterized by broad cross-reactivity

with the intestinal receptor from both mammalian and avian sources. Since the monoclonal antibody IVG recognized all of the 1,25-$(OH)_2D_3$ receptors in the crude nuclear extract of HL-60 cells (Fig 1.), immunoprecipitation using IVG was performed to increase the purity and concentration of 1,25-$(OH)_2D_3$ receptor. The enriched 1,25-$(OH)_2D_3$ receptor from HL-60 cells was detected as a single form of 53 kDa using ^{125}I- IVG. The electrophoretic mobilities of the up-regulated and down-regulated 1,25-$(OH)_2D_3$ receptor protein were not altered compared to that of the basal receptor. However, there was a 3-fold increase in the amount of receptor from HL-60 cells treated with 1,25-$(OH)_2D_3$ for 12 h (up-regulated receptor) compared to that from 2 h treatment (basal receptor) (Fig. 2). This phase was followed by the decrease in the level of 1,25-$(OH)_2D_3$ receptor to the basal level by 36 h. The induction of the increase in the amount of 1,25-$(OH)_2D_3$ receptor required new protein synthesis since cycloheximide completely inhibited the rise in the receptor level. These results are well correlated with [^{3}H]1,25-$(OH)_2D_3$ binding studies as shown in Figure 3. Throughout these studies the intensity of band from the western blot was proportional to the amount of immunoprecipitated receptor protein loaded on the SDS-polyacrylamide gel (data not shown). However, the immunoblotting assay is not generally sensitive enough for the accurate quantitative determination of receptor protein. The broad diffuse bands observed just above the receptor band or at 26 kDa may correspond to the heavy and light chains of goat anti-mouse IgG used for the immunoprecipitation since two broad diffuse bands were observed at the same position when a blank sample, containing monoclonal antibody and goat anti-mouse IgG without receptor preparation, were immunoblotted (data not shown). Previously it has been reported that vitamin D metabolites up-regulated the number of 1,25-$(OH)_2D_3$ receptor via a

receptor-mediated induction mechanism in a cultured pig kidney cell line (22). Mutant skin fibroblasts from patients with Vitamin D-dependent rickets type II, containing nonresponsive vitamin D_3 receptors, failed to exhibit the characteristic up-regulation observed in normal cells (22,47). More recently, it has been demonstrated that 1,25-$(OH)_2D_3$ can increase mRNA activity for its receptor in mouse fibroblasts (3T6), indicative of receptor autoregulation (48). These data support our hypothesis that the presence of 1,25-$(OH)_2D_3$ leads to a homologous up-regulation of 1,25-$(OH)_2D_3$ receptors in HL-60 cells.

While both the immunodetection and ligand binding methods estimated a similar increase in the amount of 1,25-$(OH)_2D_3$ receptor induction during up-regulation, they differed quantitatively for estimating the degree of down-regulation. Utilizing Western blotting, the level of the up-regulated receptor was observed to decline to basal levels by 36-48 h and remained at that level up to 72 h during continuous hormone incubation (Fig. 4). Conversely, [^{3}H]1,25-$(OH)_2D_3$ binding data showed that at 48 h and 72 h, up-regulated receptors had declined to 46% and 66% of the basal level, respectively (Fig. 5). This difference may be interpreted to mean that although receptor protein is present during down-regulation as detected by Western blots, the loss of specific [^{3}H]1,25-$(OH)_2D_3$ binding indicates that the ligand binding site is lost preferentially or at least in incapable of binding ligand. Similarly, it has been reported that the level of 1,25-$(OH)_2D_3$ receptors in human breast cancer cells undergoes homologous down-regulation by continuous hormone exposure (24). Moreover, for the estrogen receptor the disappearance of hormone receptor complex has been shown to be largely due to a loss of hormone-binding capacity of steroid hormone (processing) (49, 50).

The control of the expression of steroid hormone receptor is not well understood. The data presented here suggest that the homologous up-regulation followed by down-regulation of 1,25-$(OH)_2D_3$ receptor may represent a nuclear mechanism for the control of cellular responsiveness to hormone in the intact human target cells. In addition, these alterations might be components of the cellular differentiation process, since 1,25-$(OH)_2D_3$ is the most potent inducing agent for monocytic differentiation of HL-60 cells (8). HL-60 cells have shown to regulate other types of receptors during monocytic and granulocytic differentiation, including receptors for insulin and phorbol diester (51-53).

The immunological detection system for the 1,25-$(OH)_2D_3$ receptor employed in the present study was specific for identifying the 1,25-$(OH)_2D_3$ receptors (see Fig. 6 and 7). The XVI recognized only porcine 1,25-$(OH)_2D_3$ receptor and clearly bound to an epitope distinct from antibody IVG (38). The XVI precipitated all the receptors from the porcine intestinal nuclear extract while it did not recognize the receptors from nuclear extracts of HL-60 cells (Fig. 6). The 1,25-$(OH)_2D_3$ receptors in HL-60 cells are a single form of protein species of 53 kDa. The pig intestinal receptors are detected at 55 kDa as described previously (39). The immunoprecipitation procedure itself did not affect the electrophoretic mobility of the receptor protein since the pig intestinal receptors immunoprecipitated with either IVG or XVI have the same electrophoretic mobility as those without the immunoprecipitation. These results are consistent with the conclusions that 1,25-$(OH)_2D_3$ receptors are protein species ranging from 52 to 60 kDa and though their functional and immunological domains have been evolutionarily conserved, an inverse relationship apparently exists between phylogenic status and receptor mass (48). The data presented here clearly demonstrated that the level of 1,25-$(OH)_2D_3$

receptors in intact human target cells undergoes up-regulation followed by down-regulation upon continuous exposure to 1,25-$(OH)_2D_3$.

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Figure 1. Immunoprecipitation of [^{3}H]1,25-$(OH)_2D_3$ binding activity in the nuclear extract of pig intestine (O) and cellular extract of HL-60 (Δ). Each extract was labeled with 1.0 nM [^{3}H]1,25-$(OH)_2D_3$ in the presence or absence of a excess hormone. 200 µl of the labeled sample, containing 12 fm of specific binding activity, was then incubated with the indicated amounts of monoclonal antibody IVG for 16 h. Following immunoprecipitation with 50 µl of goat anti-mouse IgG-Sepharose, the supernatant was removed for HAP assay. The remaining pellet was counted in 4 ml of Opti-fluor scintillation fluid in a liquid scintillation counter. 100% was defined as the sum of the specific binding activity in the supernatant and precipitate.

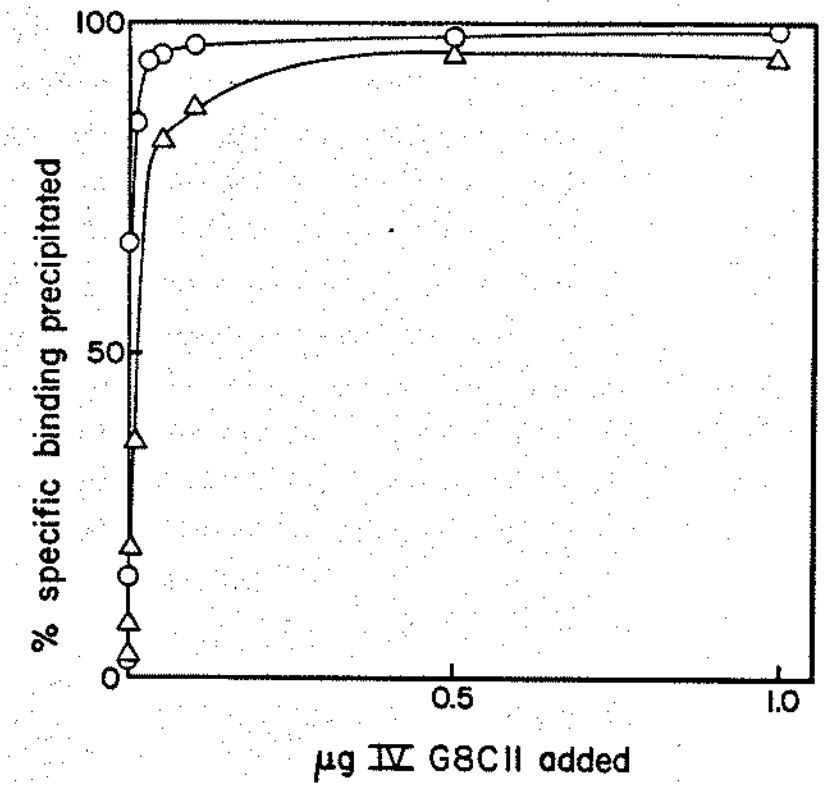

100
50
0
% specific binding precipitated
0.5
1.0
μg IV G8C11 added

Figure 2. Immunoblotting of *in vivo* occupied 1,25-$(OH)_2D_3$ receptors in HL-60 cells during the homologous regulation. Cells were treated with 2 nM 1,25-$(OH)_2D_3$ alone for 2 h (lane 1), 12 h (lane 3), and 36 h (lane 5) or in the presence of 10 μM of cycloheximide for 2 h (lane 2), 12 h (lane 4), and 36 h (lane 6) at a seeding density of 5×10^6 cells per 1 ml of SFM. The nuclear extracts were prepared and immunoprecipitated with IVG followed by the incubation with goat anti-mouse IgG-Sepharose. After the denatured receptor in SDS was electrophoresed and transferred to PVDF membrane, the membrane was probed with ^{125}I-IVG. Each lane represents the immunoprecipitated receptors from 150×10^6 cells. Molecular mass standards are shown to the left of gel; lysozyme 14.3, β-lactoglobin 18.4, carbonic anhydrase 29.0, ovalbumine 43.0, bovine serum albumin 68.0, phosphorylase β 97.4, and myosin (H-chain) 200 kDa. The arrow indicates the position of 1,25-$(OH)_2D_3$ receptor (53 kDa). The ratios of density for the bands are 1.0:0.6:3.2:0.4:1.2:0.1 from lane 1 to lane 6 as measured by Soft Laser scanning densitometer.

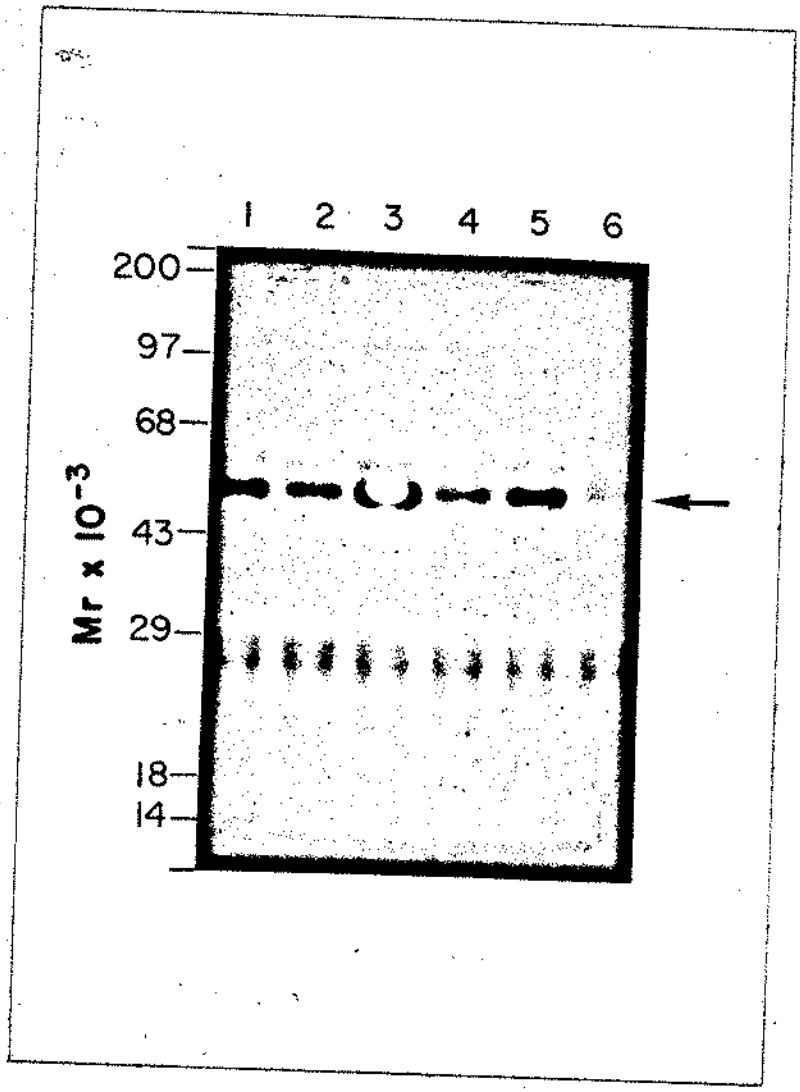

1 2 3 4 5 6
200
97
68
43
29
18
14
Mr x 10⁻³

Figure 3. Effect of cycloheximide on the time-dependent regulation of [^{3}H]1,25-$(OH)_2D_3$ uptake into intact HL-60 cells. Cells cultured in SFM were incubated in SFM with 1 nM [^{3}H]1,25-$(OH)_2D_3$ in the presence or absence of a 200-fold excess of nonradioactive hormone with (●) or without (○) 10 μM cycloheximide. Cells were placed in humidified 95% air-5% CO_2 atmosphere at 37° C for indicated times at a seeding density of 5×10^6 cells per 1 ml of SFM. Viability of cells were examined by trypan blue exclusion at the end of the incubation periods. Specifically bound [^{3}H]1,25-$(OH)_2D_3$ in total cells was quantified by the HAP batch assay. Values represent the means for four replicate wells with duplicate measurements from each well. All values of SEM are smaller than the diameter of symbols.

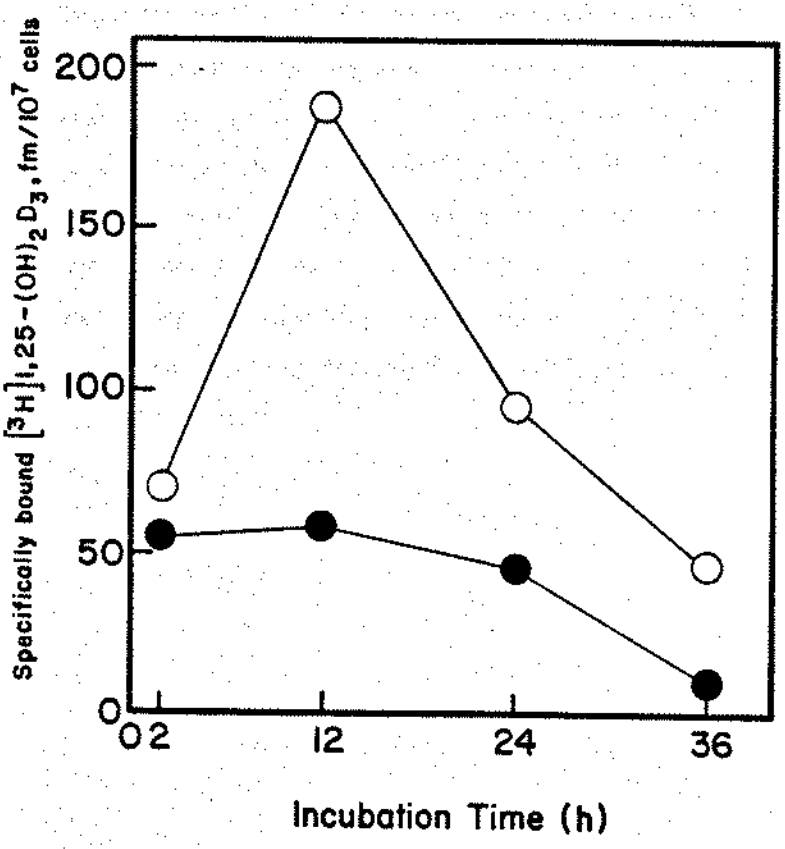

Specifically bound [3H]1,25-(OH)2D3, fm/10^7 cells
200
150
100
50
0
0 2
12
24
36
Incubation Time (h)

Figure 4. Examination of the down-regulated receptor level from HL-60 cells exposed to hormone for up to 72 h. HL-60 cells in SFM were incubated with 2 nM 1,25-$(OH)_2D_3$ for 2 (lane 1), 12 (lane 2), 24 (lane 3), 48 (lane 4), and 72 h (lane 5) at a seeding density of 5×10^5 cells per ml. To the cells with 48 and 72 h incubation, fresh hormone (2 nM) was added 24 h prior to the harvest. After each incubation period, nuclear extracts from HL-60 cells were immunoprecipitated with IVG, electrophoresed and then immunoblotted with ^{125}I-IVG as described in Fig. 3. Each lane represents the immunoprecipitated receptors from 200×10^6 HL-60 cells. Lane 6 represents 200 μg of protein of porcine nuclear extract without immunoprecipitation. Molecular mass standards are shown to the left of the gel. The arrows indicate the positions of 1,25-$(OH)_2D_3$ receptor from HL-60 cells (53 kDa) and pig intestine (55 kDa).

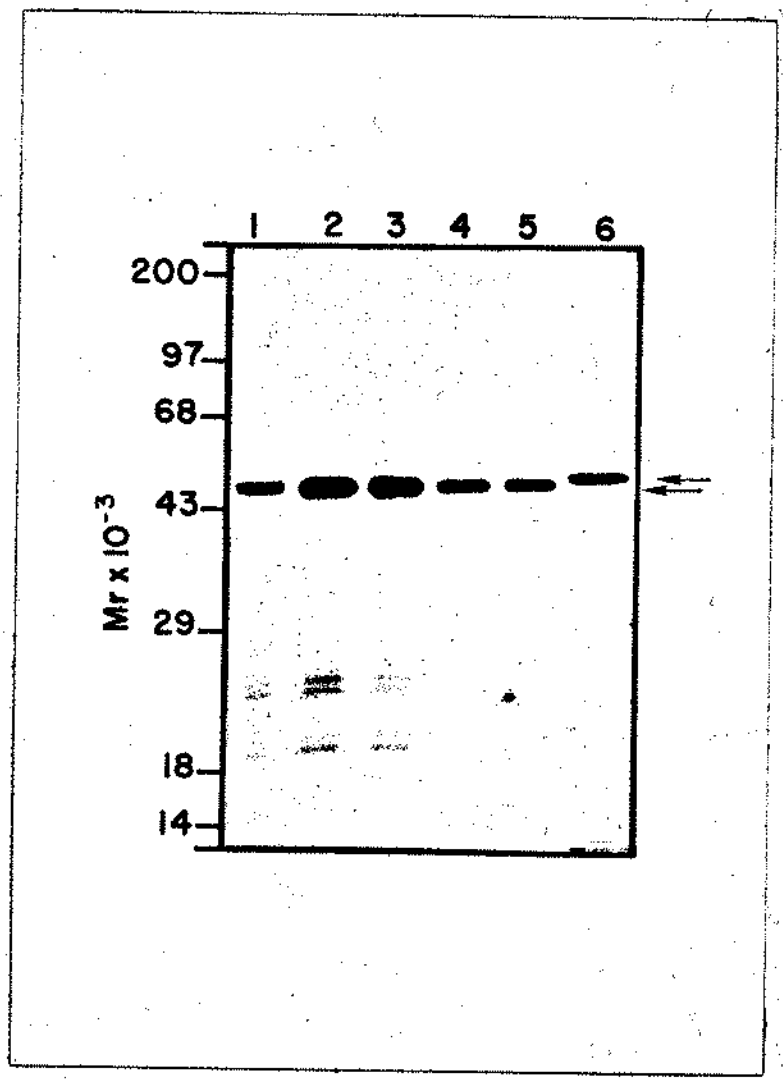

1
2
3
4
5
6
200
97
68
43
29
18
14
Mr x 10⁻³

Figure 5. Time-dependent regulation of [^{3}H]1,25-$(OH)_2D_3$ uptake into intact HL-60 cells. HL-60 cells (5×10^5 cells/ml) were labeled with 2 nM [^{3}H]1,25-$(OH)_2D_3$ in the presence or absence of a 200-fold excess nonradioactive hormone for up to 72 h. Fresh hormone (2 nM [^{3}H]1,25-$(OH)_2D_3$ with or without a excess nonradioactive hormone) was added to the cells with 48 and 72 h-incubation 24 h prior to harvest. After the incubation periods, total cellular specific hormone binding activities were measured by HAP assay.

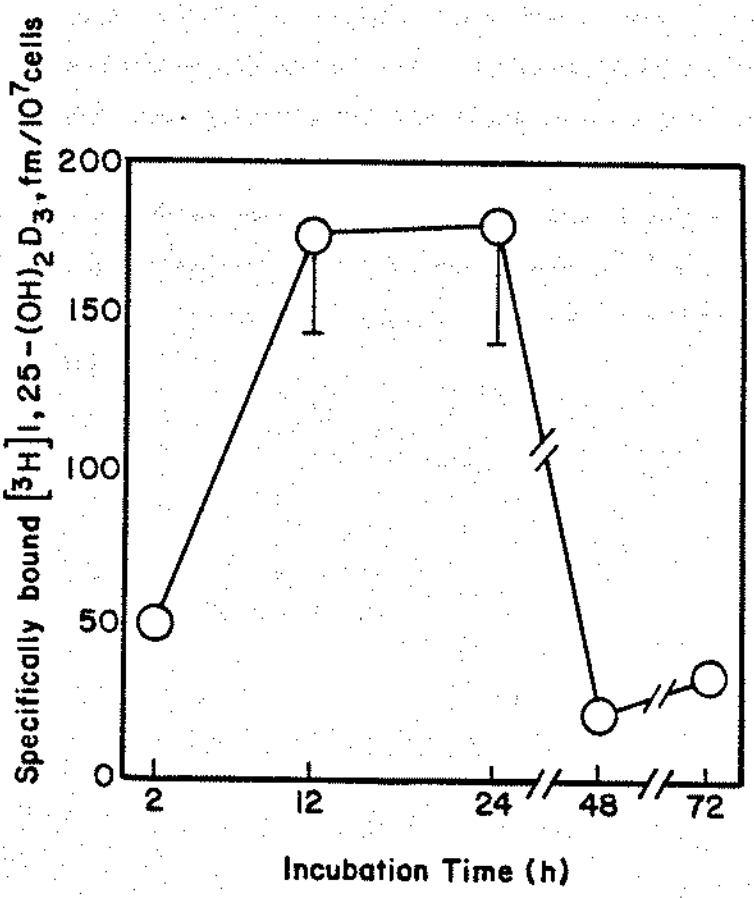

Specifically bound [3H]1,25-(OH)2D3, fm/10^7cells
200
150
100
50
0
2
12
24
48
72
Incubation Time (h)

Figure 6. Immunoprecipitation of [^{3}H]1,25-$(OH)_2D_3$ binding activity by monoclonal antibodies, IVG or XVI. The nuclear extract of pig intestine was incubated with 1 nM [^{3}H]1,25-$(OH)_2D_3$ with or without a 200-fold excess nonradioactive hormone in the presence of IVG (open bar) or XVI (hatched bar) at 4° C for 16 h. The nuclear extract from HL-60 cells treated with [^{3}H]1,25-$(OH)_2D_3$ for 2 h was also incubated with IVG or XVI. The sufficient amount of monoclonal antibody was added to react all the binding activities in the nuclear extracts; 10 ng of antibodies was added to immunoprecipitate 1 fm of each receptor in extracts (Fig.1). The immunoprecipitation efficiencies were determined as described in Fig. 1.

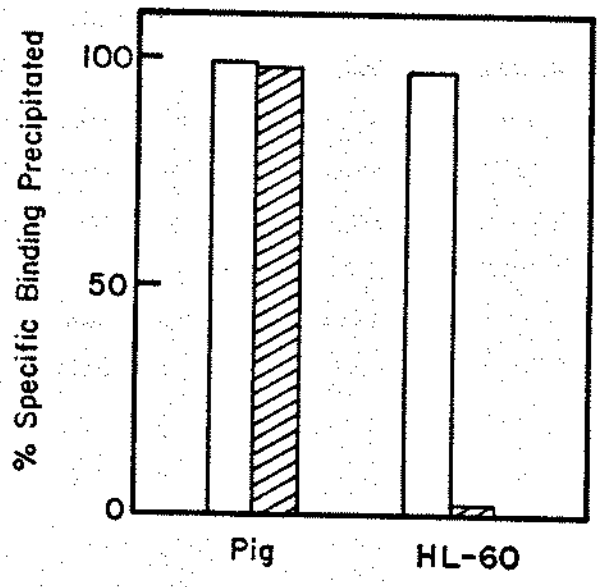
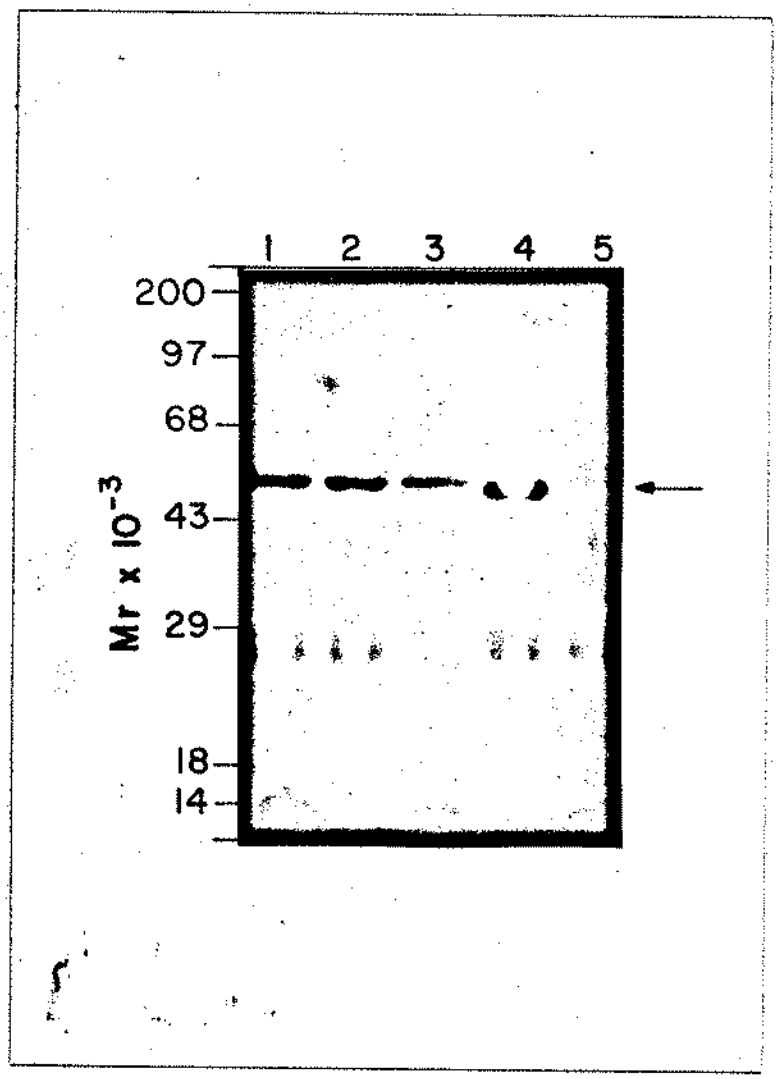

% Specific Binding Precipitated
100
50
0
Pig
HL-60

Figure 7. Immunoblotting of 1,25-$(OH)_2D_3$ receptor in the nuclear extracts of HL-60 cells and pig intestine. The nuclear extract from porcine intestine (550 μg of protein) was incubated with 2 nM [^{3}H]1,25-$(OH)_2D_3$ in the presence of IVG (lane 1) or XVI (lane 2). After HL-60 cells were incubated with 2 nM 1,25-$(OH)_2D_3$ for 2 h at 37° C, nuclear extracts were immunoprecipitated with IVG (lane 4) or XVI (lane 5). These steps were followed by the incubation with goat anti-mouse IgG-Sepharose at 4° C. After the immunoprecipitated receptors were electrophoresed, they were transferred to PVDF membrane which were subsequently probed with ^{125}I-IVG. Lane 3 represents 200 μg of protein of porcine nuclear extract without immunoprecipitation; lanes 4 and 5 are immunoprecipitated receptors from 150 × 10^6 HL-60 cells. Molecular mass standards are shown to the left of the gel. The arrow indicate the positions of 1,25-$(OH)_2D_3$ receptor from HL-60 (53 kDa).


1
2
3
4
5
200
97
68
43
29
18
14
Mr x 10-3

CHAPTER V

IMMUNOLOGICAL CHARACTERIZATION OF THE REGULATION OF 1,25-DIHYDROXYVITAMIN D_3 RECEPTORS IN HUMAN PROMYELOCYTIC LEUKEMIC CELLS (HL-60) DURING CELLULAR DIFFERENTIATION

Youngsook Lee, Hector F. DeLuca and William S. Mellon

(Submitted for publication)

SUMMARY

The regulation of 1,25-dihydroxyvitamin D_3, 1,25-$(OH)_2D_3$, receptors in intact target cells was examined in association with the cellular differentiation using a human promyelocytic leukemic cell line, HL-60, as a model system. The HL-60 cells regulated the level of 1,25-$(OH)_2D_3$ receptor as cells were incubated with monocytic inducers of HL-60, 10 nM phorbol 12-myristate 13-acetate (PMA) or 0.5 nM [^{3}H]1,25-$(OH)_2D_3$. An increase in the specific [^{3}H]1,25-$(OH)_2D_3$ binding was observed by 12 h incubation with PMA or [^{3}H]1,25-$(OH)_2D_3$, followed by a decrease by 24-48 h. However, the granulocytic inducers of HL-60 cells, retinoic acid (10^{-8} M and 10^{-6} M) and 1.2% dimethyl sulfoxide, failed to regulate the specific [^{3}H]1,25-$(OH)_2D_3$ binding. The immunoblot assay using the monoclonal antibody to 1,25-$(OH)_2D_3$ receptor, IVG8C11, indicated the induction of an increase in the amount of 1,25-$(OH)_2D_3$ receptor, followed by a decrease without alterations in the electrophoretic mobility of the receptor (53 kDa) as cells were treated with 10 nM PMA. The present study suggests that the regulation of 1,25-$(OH)_2D_3$ receptors in HL-60 cells might be associated with the monocytic differentiation process.

INTRODUCTION

The mode of action of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), the biologically active metabolite of vitamin D_3, within target tissues appears to be similar to that of steroid hormone in that 1,25-$(OH)_2D_3$ first binds to a specific high affinity intracellular receptor which consequently induces the modulation of gene expression (1). Among various inducers such as vitamins, tumor promoters, highly polar compounds, and anticancer agents (2,3), 1,25-$(OH)_2D_3$ has been shown to be the most potent monocytic inducer of the differentiation of human promyelocytic leukemic cell line, HL-60 (4). The action of 1,25-$(OH)_2D_3$ on HL-60 cells is also believed to be receptor mediated since a receptor for 1,25-$(OH)_2D_3$ was identified in HL-60 cells and has the same physical characteristics of the receptor in the well characterized 1,25-$(OH)_2D_3$ target tissues, i.e., the intestine (5), and the ability of various analogs to cause HL-60 differentiation correlates well with their binding affinity to the 1,25-$(OH)_2D_3$ receptor (6,7).

The alteration of hormone receptor level is an important mechanism for target cell responsiveness to hormones (8). In endocrine target cells, evidence exists for regulation of multiple receptor systems, both steroids and peptides, by homologous or heterologous hormone (9-11). Cells are known to regulate their receptor populations in response to many external and internal stimuli, including differentiation. The specific 1,25-$(OH)_2D_3$ binding activity was stimulated by other hormones during the induction of mammary functional differentiation (12). Among the developmental changes occurring in the intestine, the appearance of 1,25-$(OH)_2D_3$ binding activity in postnatal rat intestine is initiated by glucocorticoids (13). The number of triiodothyronine receptor sites approximately doubled during adipocyte differentiation (14). Little is known, however, about how these events

interact during the process of differentiation or which mechanisms are directly involved in this process. Having observed an increase in specific binding of 1,25-$(OH)_2D_3$ receptors in HL-60 cells followed by a decrease upon continuous exposure to 1,25-$(OH)_2D_3$ (15), the possibility that the receptor regulation might be a component of cellular differentiation was examined. Since HL-60 can be induced to undergo differentiation toward either a mature granulocyte or monocyte depending on the inducers used (3,5), these bipotent cells were used as a model to examine whether 1,25-$(OH)_2D_3$ receptors are regulated as cells differentiate *in vitro*. HL-60 cells were cultured in a serum-free medium (SFM) in order to eliminate the undesirable factors present in serum (16).

MATERIALS AND METHODS

Materials. 1α,25-dihydroxyvitamin D_3, radiolabeled with tritium at the 26,27-methyl groups ([^{3}H] 1,25-$(OH)_2D_3$, 160 Ci/mmol), was obtained from Du Pont, NEN Research Products (Boston, MA). Nonradioactive 1,25-$(OH)_2D_3$ was a gift from Dr. M. Uskokovic of Hoffmann-LaRoche, Inc. (Nutley, NJ). Determinations of purity and concentration of 1,25-$(OH)_2D_3$ were achieved by UV absorption spectroscopy using an extinction coefficient (E = 264) of 18,200 M^{-1} cm^{-1}. Hydroxylapatite, acrylamide and bis-acrylamide were purchased from BIO-RAD (Richmond, CA), Sephadex G-25 (med), particle size 50-150 μm, from Pharmacia, Inc. (Piscataway, NJ), glycine and sodium dodecyl sulfate (SDS) from Schwarz/Mann Biotech (Division of ICN Biomedicals, Inc., Cleveland, OH). Prestained protein molecular weight standards (molecular weight ranges from 14,300-200,000) were obtained from Bethesda Research Laboratories Life Technologies, Inc., (Gaithersburg, MD).

Cell Culture. The human promyelocytic leukemic cells, HL-60, were maintained in RPMI-1640 medium buffered with 25 mM Hepes (Sigma Chemical Co., St. Louis, MO) containing 100 units/ml penicillin and 0.5 μg/ml streptomycin. This basal medium was supplemented with a defined supplement of insulin from bovine pancreas (5 μg/ml), human transferrin (5 μg/ml), sodium selenite (5 ng/ml), and BSA (fatty acid free, 0.5 mg/ml) (Sigma Chemical Co., St. Louis, MO), designated as a serum free medium, SFM (16). HL-60 cells were grown in SFM at 37° C in a humidified 95% air-5% CO_2 atmosphere and subcultured with 4 day intervals at a seeding density of 3×10^5 cells per ml. Cell proliferation was assessed by counting cells in a hemocytometer and cell viability was determined by trypan blue dye exclusion.

Preparation of 1,25-$(OH)_2D_3$ Receptors. Receptors for 1,25-$(OH)_2D_3$ from HL-60 cells were prepared as described previously (16). Briefly, HL-60 cells cultured in SFM were incubated with 2 nM 1,25-$(OH)_2D_3$ in SFM at 37° C in humidified 95% air-5% CO_2 atmosphere for various times. The following procedures were done at 4° C. After cells were washed twice in PBS, they were resuspended and incubated for 20 min in TEDM buffer with intermittent vortexing followed by homogenization using a Tissuemizer type SDT (Tekmar Co., Cincinnati, OH) for 3 sec at a speed setting of 50. The homogenate was centrifuged for 10 min at 800 g to obtain crude nuclear fractions. The crude nuclear pellet was incubated in TEDMK-0.3 for 30 min followed by centrifugation at 105,000 g for 45 min in a Beckman L5-50B ultracentrifuge using a type Ti-50 rotor (Beckman Instruments, Palo Alto, CA). The supernatant was utilized as the source of occupied calcitriol receptors in HL-60 cells for electrophoresis experiments. The nuclear extracts from porcine intestine were prepared as described previously (17).

Measurement of Total Cellular Receptors. The amount of total cellular receptors for 1,25-$(OH)_2D_3$ was determined in HL-60 cells as described previously (16). Briefly, cells in SFM were incubated with various concentrations of [^{3}H]1,25-$(OH)_2D_3$ in the presence or absence of a 200-fold excess of nonradioactive hormone. After the each incubation period, cells were washed twice with PBS and resuspended in TEDM buffer for 20 min at 4° C with intermittent vortexing. Aliquots of cells in TEDM buffer were assayed for receptor-bound [^{3}H]1,25-$(OH)_2D_3$ by hydroxylapatite (HAP) assay (18) with slight modification (16). Specific binding was estimated as the difference between total binding ([^{3}H]1,25-$(OH)_2D_3$ alone) and nonspecific binding (with a 200-fold excess nonradiolabeled hormone). The radioactivity was measured by liquid scintillation spectrometry (Packard Tri-Carb Spectrometer, model Prias PLD) using Opti-fluor Scintillation fluid (Packard, Downers Grove, IL) with an efficiency for tritium of 30%.

Immunoprecipitation. The monoclonal antibody to the porcine intestinal 1,25-$(OH)_2D_3$ receptor used in this work, IVG8C11 (IVG), were described previously (19). The nuclear extracts from HL-60 cells treated with 1,25-$(OH)_2D_3$ or [^{3}H]1,25-$(OH)_2D_3$ were incubated with 5-10 ng of monoclonal antibodies to immunoprecipitate 1 fm of 1,25-$(OH)_2D_3$ receptor with continuous shaking for 16 h at 4° C. The pig nuclear extract was incubated simultaneously with [^{3}H]1,25-$(OH)_2D_3$ in the presence or absence of a 200-fold excess of nonradiolabeled hormone and with monoclonal antibodies. For precipitation, 50 µl of goat anti-mouse IgG-Sepharose (Organon Teknika Corporation, West Chester, PA) in a 50% slurry in PBS was added to each tube and incubated for 1 h at 4° C with continuous shaking. Following incubation, the tubes were centrifuged at 1,500 g for 5 min,

the supernatant removed for binding assay or discarded, and the Sepharose pellets washed twice by resuspension with 1 ml of PBS containing 0.5% triton x100 followed by centrifugation. To determine the efficiency of immunoprecipitation, the Sepharose beads incubated with [^{3}H]1,25-$(OH)_2D_3$ labeled receptor were transferred to scintillation vials with two 200-µl washes of ethanol and 4 ml of Opti-fluor scintillation fluid added. The radioactivity was measured as described above. To electrophorese the immunoprecipitated receptors, the Sepharose beads incubated with occupied 1,25-$(OH)_2D_3$ receptors were boiled for 5 min in 1 vol of SDS-denaturing buffer. After the tubes were centrifuged at 200 g for 10 min at 25° C, the supernatants were electrophoresed on 9% SDS-polyacrylamide gels (1.5 mm spacer) as described by Laemmli (20).

Immunoblot. For use in immunoblotting experiments, purified monoclonal antibody, IVG, was iodinated with ^{125}I-Bolton-Hunter reagent (NEN Research Products) as described previously (21). Briefly, 10 µg of IVG in 10 µl of PBS were reacted with ^{125}I-Bolton-Hunter reagent (1 mci) for 2 h on ice. To stop the reaction, 0.25 ml of glycine in 0.1M borate (pH 8.5) was added for 5 min, then 0.25 ml of PBS containing 0.25%gelatin was added to the reaction vial. The monoclonal antibody coupled with ^{125}I was separated from free ^{125}I using Sephadex G-25 (medium) column, which was loaded with 1 ml of 3% (w/v) bovine serum albumin in PBS and then equilibrated with PBS containing 0.25% gelatin. Fractions were collected (0.5 ml) and 5 µl-aliquots were removed from each fraction to determine the radioactivity in a Prias PLD scintillation spectrometer (Packard) using a channel optimized for determination of ^{125}I. After the peak fractions were combined, 10 µl-aliquots were removed to measure radioactivity. ^{125}I-IVG was examined using deoxycholate-TCA precipitation method (80% of the

radioactivity was precipitated by incubation with 100 µl of 0.15% deoxycholate and 100 µl of 72% TCA.) After electrophoresis, the gels were equilibrated in transfer buffer for 15 min and transferred electrophoretically to polyvinylidene difluoride (PVDF) membrane (Milipore Corporation, Bedford, MA) at 145 mA for 16 h using a Transphor electrophoresis unit (Hoefer Scientific Instrument, San Francisco, CA). The PVDF membrane was prewet in 100% methanol for 1-2 sec, rinsed in water for 5 min and then equilibrated with transfer buffer for 10-15 min prior to use in blotting. After electrophoretic transfer, the membranes were washed with PBS for 10 min to remove transfer buffer, blocked with 5% blotto for 2 h at room temperature, and rinsed with 0.5% blotto for 2-5 min. The membranes were then incubated with 3×10^5 cpm/ml of ^{125}I-IVG in 0.5% blotto for 3 h. After the membranes were washed five times for 10 min each with 0.5% blotto and rinsed in PBS, they were air-dried, and autoradiographed using Kodak XAR2 film with Du Pont Cronex Quanta III enhancing screens (Du Pont, New England Nuclear) at -70° C.

Buffers. The compositions of buffers used in these experiments are as follows: Phosphate-buffered saline (PBS), 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 8.0, 137 mM NaCl, 2.7 mM KCl; Tris-buffered saline with Tween 20 (TBST), 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20; Blocking buffer (Blotto), 5% (w/v) Carnation non-fat dry milk and 0.02% (W/V) NaN_3 in TBST; SDS-denaturing buffer, 0.125 M Tris, pH 6.8, 4% SDS (w/v), 20% (v/v) glycerol, 0.002% Bromophenol blue, 10% mercaptoethanol; Gel running buffer, 0.025 M Tris, pH 8.3, 0.192 M glycine, 0.1% SDS; transfer buffer, 0.025 M Tris, pH 8.3, 0.192 M glycine, 0.1% (w/v) SDS, 20% (v/v) methanol; TEDM, 50 mM Tris-HCl,

pH 7.5, 1.5 mM EDTA, 5 mM dithiothreitol, 10 mM sodium molybdate; TEDMK-0.3, TEDM buffer with 0.3 M KCl

RESULTS

HL-60 cells cultured in SFM were treated with monocytic inducers or granulocytic inducers for up to 48 h. At the indicated time, cells were harvested to measure the specific [^{3}H]1,25-$(OH)_2D_3$ binding in intact cells. As Fig. 1.A shows, 10 nM PMA, a monocytic inducer, increased the specific [^{3}H]1,25-$(OH)_2D_3$ binding observed by 12 h. This phase was followed by a decrease in the specific [^{3}H]1,25-$(OH)_2D_3$ binding observed by 24-48 h. The regulation of [^{3}H]1,25-$(OH)_2D_3$ binding by PMA was in a similar manner to that by 1,25-$(OH)_2D_3$. 1,25-$(OH)_2D_3$ was unable to compete for the phorbol diester binding sites as measured by [^{3}H]phorbol-12,13-dibutyrate binding (22).

The cells treated with retinoic acid or dimethyl sulfoxide, known granulocytic inducers of HL-60 cells, did not exhibit the regulation of specific [^{3}H]1,25-$(OH)_2D_3$ binding in HL-60 cells (Fig. 1.B). At the concentrations of inducers used, HL-60 cells were induced to differentiate towards the monocytic or granulocytic pathway (3, 5).

The regulation of specific [^{3}H]1,25-$(OH)_2D_3$ uptake into HL-60 cells by PMA was examined using immunological techniques. Since IVG precipitated all the 1,25-$(OH)_2D_3$ receptors in the crude nuclear extract of HL-60 with a similar efficiency to that of porcine intestine (23), immunoprecipitation was performed to purify and concentrate the 1,25-$(OH)_2D_3$ receptor from the nuclear extract of HL-60 cells. The HL-60 cells in SFM were treated with 10 nM PMA or 10 nM 4α-phorbol-12,13-didecanoate (4α-P), an analogue incapable of stimulating protein

kinase C, to ascertain the specific effect of PMA on the 1,25-$(OH)_2D_3$ receptor regulation. As shown in Fig. 2, the 1,25-$(OH)_2D_3$ receptor was detected as a single form of 53 kDa protein calculated from this and other gels and its electrophoretic mobility was not altered by PMA-treatment. However, the amount of 1,25-$(OH)_2D_3$ receptor detected by ^{125}I-IVG was increased by 12 h as compared to those with 2 hour-treatment and then decreased slightly by 24 h (lane 1 to 3). The level of receptors in the cells treated with 4α-P were increased at 12 and 24 h compared to the basal level. The ratios of densities of the bands are 1.0:2.5:1.7:0.5:1.0:1.6 for the cells treated with PMA for 2, 12, and 24 h and with 4α-P for 2, 12, and 24 h, respectively as measured by Soft Laser scanning densitometer, model SL-504-XL (Biomed Instruments, Inc., Fullerton, CA). These data correlate well with the results of [^{3}H]1,25-$(OH)_2D_3$ binding studies in Fig. 1.

The specific [^{3}H]1,25-$(OH)_2D_3$ binding activity for cells treated with PMA was compared to those treated with 4α-P (Fig. 3). The specific [^{3}H]1,25-$(OH)_2D_3$ was increased by 12 h incubation with PMA followed by the decrease in [^{3}H]1,25-$(OH)_2D_3$ binding by 24 h, while the specific [^{3}H]1,25-$(OH)_2D_3$ binding was also increased by 12 and 24 h with 4α-P as compared to the basal level. The phenotypic changes associated with differentiation were examined for 4α-P. The nitroblue tetrazolium (NBT) reduction assay, which measures the ability of cells for the production of superoxide (24), indicated that 4α-P did not induce the differentiation of HL-60 cells. In addition, cells treated with 4α-P did not adhere to the bottom of flasks, while cells with PMA mostly adhered to the flasks (data not shown). Cells were induced to differentiate by incubation with 10 nM 1,25-$(OH)_2D_3$ for 3 days,

indicating a normal differentiational response of HL-60 cells cultured in SFM to inducing agents.

DISCUSSION

The 1,25-$(OH)_2D_3$ receptors in HL-60 cells became saturated with 0.5-1.0 nM [^{3}H]1,25-$(OH)_2D_3$ within 1 h and the continuous exposure of cells to hormone resulted in an increase in specific hormone binding followed by a loss of hormone binding in a time-dependent manner (15). The regulation of [^{3}H]1,25-$(OH)_2D_3$ binding by PMA was in a similar manner to that by 1,25-$(OH)_2D_3$. Like 1,25-$(OH)_2D_3$, phorbol esters induce HL-60 cell differentiation at low concentrations and they have an integral effect on cell growth and proliferation (25). The monocyte-macrophage phenotype induced by 1,25-$(OH)_2D_3$ in the HL-60 cells was found to be similar but not identical to that induced by PMA (22). Protein kinase C (the Ca^{++}/phospholipid-dependent enzyme) is the proposed receptor for tumor-promoting phorbol esters (26). Since 1,25-$(OH)_2D_3$ modulates cell Ca^{++} content and phosphatidylserine synthesis (27), agents that are involved in protein kinase C activation, and since phorbol esters and 1,25-$(OH)_2D_3$ induce differentiation, it seems likely that the mechanisms of differentiation between 1,25-$(OH)_2D_3$ and phorbol esters are related.

The cells treated with retinoic acid or dimethyl sulfoxide, known granulocytic inducers of HL-60 cells, did not exhibit the regulation of specific [^{3}H]1,25-$(OH)_2D_3$ binding in HL-60 cells (Fig. 1.B). These data suggest that the regulation of 1,25-$(OH)_2D_3$ receptor in HL-60 cells might be associated with the monocytic differentiation process. An increase in insulin binding is associated with the appearance of markers of monocytic differentiation, while decreased levels of

binding occur during myeloid differentiation of HL-60 cells or U-937 cells, a monocytic cell line (28). HL-60 cells have been shown to increase the number of [^{3}H]phorbol 12, 13-dibutyrate binding sites as they differentiate *in vitro* into mature granulocytes by dimethyl sulfoxide and into monocytes by 1,25-$(OH)_2D_3$ (29). These reports support our observations of the 1,25-$(OH)_2D_3$ receptor regulation in intact target cells in association with the monocytic differentiation pathway.

However, conclusions regarding the characterization and/or quantification of unoccupied or potentially non-hormone binding forms of the receptor cannot be drawn from these ligand binding studies. The availability of monoclonal antibodies to the 1,25-$(OH)_2D_3$ receptor enables the study of this protein independent of hormone-binding activity (19,30). The regulation of specific [^{3}H]1,25-$(OH)_2D_3$ uptake into HL-60 cells by PMA was examined using immunological techniques. The monoclonal antibody raised against the 1,25-$(OH)_2D_3$ receptor in porcine intestines (19) was utilized for the immunoprecipitation and immunoblot assay. The monoclonal antibody, IVG8C11, is characterized by its broad cross-reactivity with the receptors from both mammalian and avian sources. The HL-60 cells in SFM were treated with 10 nM PMA or 10 nM 4α-phorbol-12,13-didecanoate (4α-P), an analogue incapable of stimulating protein kinase C and therefore presumed to be biologically inactive (31), to ascertain the specific effect of PMA on the 1,25-$(OH)_2D_3$ receptor regulation. The immunoprecipitated 1,25-$(OH)_2D_3$ receptor was detected as a single form of 53 kDa protein calculated from this and other gels and its electrophoretic mobility was not altered by PMA-treatment. However, the amount of 1,25-$(OH)_2D_3$ receptor detected by ^{125}I-IVG was increased by 12 h as compared to those with 2 hour-treatment and then decreased slightly by 24 h (Fig. 2). The level of receptors in the cells treated with 4α-P were increased

unexpectedly at 12 and 24 h compared to the basal level. The ratios of densities of the bands are 1.0:2.5:1.7:0.5:1.0:1.6 for the cells treated with PMA for 2, 12, and 24 h and with 4α-P for 2, 12, and 24 h, respectively as measured by Soft Laser scanning densitometer, model SL-504-XL (Biomed Instruments, Inc., Fullerton, CA). 4α-P did not affect the differentiation of HL-60 cells while increases in specific hormone binding and amount of receptor protein was observed for cells treated with 4α-P. Since 4α-P is considered to be a biologically inactive phorbol ester analogue due to its inability to stimulate protein kinase C, these data might suggest that the regulation of 1,25-$(OH)_2D_3$ receptor might not be mediated solely by protein kinase C, while the differentiation of HL-60 cells is mediated by protein kinase C. Although the immunoblotting assay is not generally sensitive for the accurate quantification of the protein, these data correlate well with the results of [^{3}H]1,25-$(OH)_2D_3$ binding studies in Fig. 1.

We have observed that the increase in the amount of 1,25-$(OH)_2D_3$ receptor was followed by the decrease in a time-dependent manner upon continuous exposure to 1,25-$(OH)_2D_3$ in intact HL-60 cells using immunoblot assay (23) and that the physical/chemical characteristics of 1,25-$(OH)_2D_3$ receptors were not altered as indicated by the results of sucrose density gradient, DNA-cellulose chromatography, and saturation analysis (15). The specific [^{3}H]1,25-$(OH)_2D_3$ binding activity for cells treated with PMA was compared to those treated with 4α-P (Fig. 3). The specific [^{3}H]1,25-$(OH)_2D_3$ was increased by 12 h incubation with PMA followed by the decrease in [^{3}H]1,25-$(OH)_2D_3$ binding by 24 h. The specific [^{3}H]1,25-$(OH)_2D_3$ binding was also increased by 12 and 24 h with 4α-P as compared to the basal level, which correlated well with the result from immunoblotting assay (Fig. 2). The phenotypic changes associated with

differentiation were examined for 4α-P. The nitroblue tetrazolium (NBT) reduction assay, which measures the ability of cells for the production of superoxide (24), indicated that 4α-P did not induce the differentiation of HL-60 cells. In addition, cells treated with 4α-P did not adhere to the bottom of flasks, while cells with PMA mostly adhered to the flasks (data not shown). Cells were induced to differentiate by incubation with 10 nM 1,25-$(OH)_2D_3$ for 3 days, indicating a normal differentiational response of HL-60 cells cultured in SFM to inducing agents.

This study attempts to provide insight into the mechanism of the monocytic differentiation of HL-60 cells by examining the regulation of 1,25-$(OH)_2D_3$ receptor. The data presented here suggest that the regulation of 1,25-$(OH)_2D_3$ receptors in HL-60 cells might be components of the cellular differentiation process induced by monocytic inducers. The homologous or heterologous alterations of 1,25-$(OH)_2D_3$ receptor number in target cells are found under many conditions. However, the reason or mechanism for this change in the amount of 1,25-$(OH)_2D_3$ receptor in HL-60 cells is not apparent. It could be speculated from the present study and others (12-14,21,22) that the increase in the number of 1,25-$(OH)_2D_3$ receptor augments the cell's sensitivity to external hormonal stimuli, which may be required for the monocytic differentiation but not for the granulocytic differentiation. The loss of specific hormone binding may represent a control mechanism for the termination of the effect of the increased hormone-receptor complex.

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Table 1.

Functional changes in HL-60 cells induced by phorbol ester analogue

Treatment	NBT reduction (%)
control (vehicle)	6.7
4α-phorbol 12,13-didecanoate (10 nM)	7.7
1,25-$(OH)_2D_3$ (10 nM)	81.0

Cells were inoculated in SFM at 3×10^5 cells/ml and treated for 3 days with one of the compounds at 37° C. To perform the NBT reduction assay, cells (2×10^6 cells per 1 ml of SFM) were incubated with 1 ml of 0.2% NBT in PBS containing 200 ng PMA at 37° C for 30 min. The values show the mean percentage of the total cells having blue-black deposits from four measurements. At least 200 cells were counted for each measurement using a hemocytometer.

Figure 1. Effect of monocytic (A) and granulocytic (B) inducers on the time-dependent regulation of specific binding of [^{3}H]1,25-$(OH)_2D_3$ in HL-60 cells. Cells were incubated with 0.5 nM [^{3}H]1,25-$(OH)_2D_3$ (O) in the presence or absence of a 200-fold excess of nonradiolabeled hormone for up to 48 h. After cells were incubated with 10 nM PMA (●), 1.2% DMSO (Δ), or retinoic acid (10^{-8} M, ■; 10^{-6} M, ❑) for up to 48 h, specific binding of [^{3}H]1,25-$(OH)_2D_3$ was assessed by labeling cells (5×10^6 cells) with 0.5 nM [^{3}H]1,25-$(OH)_2D_3$ in the presence or absence of a 200-fold excess nonradiolabeled hormone for 1-2 h at 37° C. Specifically bound [^{3}H]1,25-$(OH)_2D_3$ was quantified by an HAP assay. Values represent the mean ± SE for two to ten replicate wells with duplicate measurement from each well.

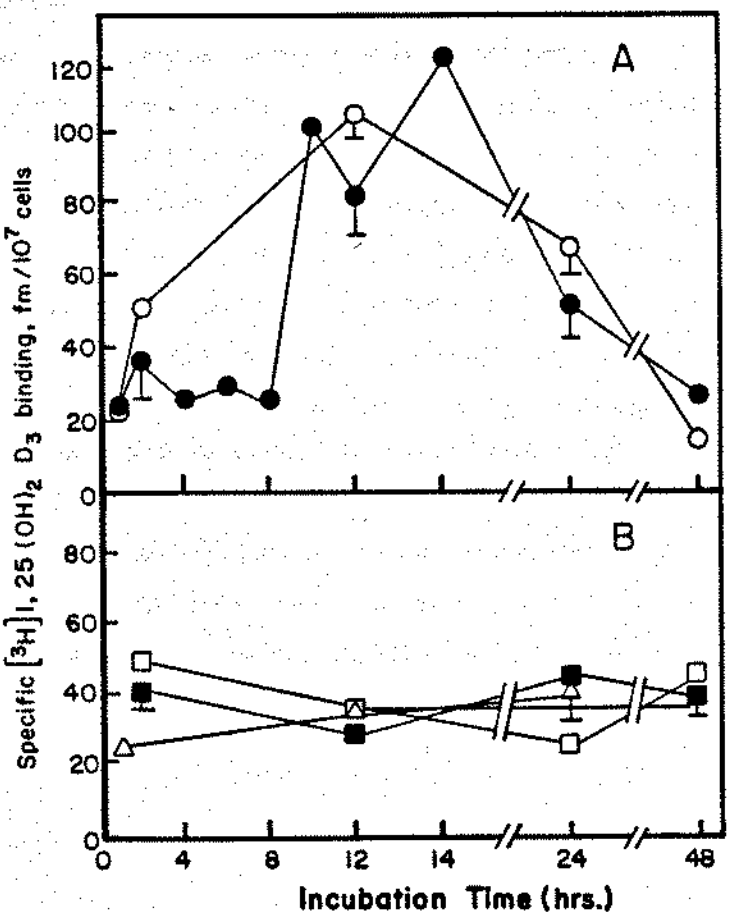

A
B
120
100
80
60
40
20
0
80
60
40
20
0
0
4
8
12
14
24
48
Incubation Time (hrs.)
Specific [3H]1, 25 (OH)2 D3 binding, fm/10^7 cells

Figure 2. Immunoblotting assay to characterize 1,25-$(OH)_2D_3$ receptors in HL-60 cells treated with PMA. HL-60 cells (5×10^5 cells per 1 ml of SFM) were incubated with 10 nM PMA or 10 nM 4α-P for 2, 12 and 24 h at 37° C. After each incubation period, cells were treated with 2 nM 1,25-$(OH)_2D_3$ for 2 h at 37° C and nuclear extracts were prepared and immunoprecipitated with IVG8C11. The immunoprecipitation efficiency for the HL-60 cell receptors was about 85-90%. After the immunoprecipitated receptor was electrophoresed on SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes electrophoretically, the membranes were probed with ^{125}I-IVG8C11. Lanes 1, 2 and 3 represent the immunoprecipitated receptor from cells treated with PMA for 2, 12, and 24 h, respectively. Lanes 4, 5 and 6 represent the immunoprecipitated receptor from cells treated with 4α-P for 2, 12 and 24 h, respectively. Each lane (lane 1 to 6) represents the immunoprecipitated receptor from 200×10^6 HL-60 cells. Lane 7 represents 200 μg protein of porcine intestinal nuclear extract without immunoprecipitation. Molecular mass standards are shown to the left of the gel. The arrows indicate the positions of 1,25-$(OH)_2D_3$ receptor from HL-60 cells (53 kDa) and pig intestine (55 kDa). These data were reproducible.

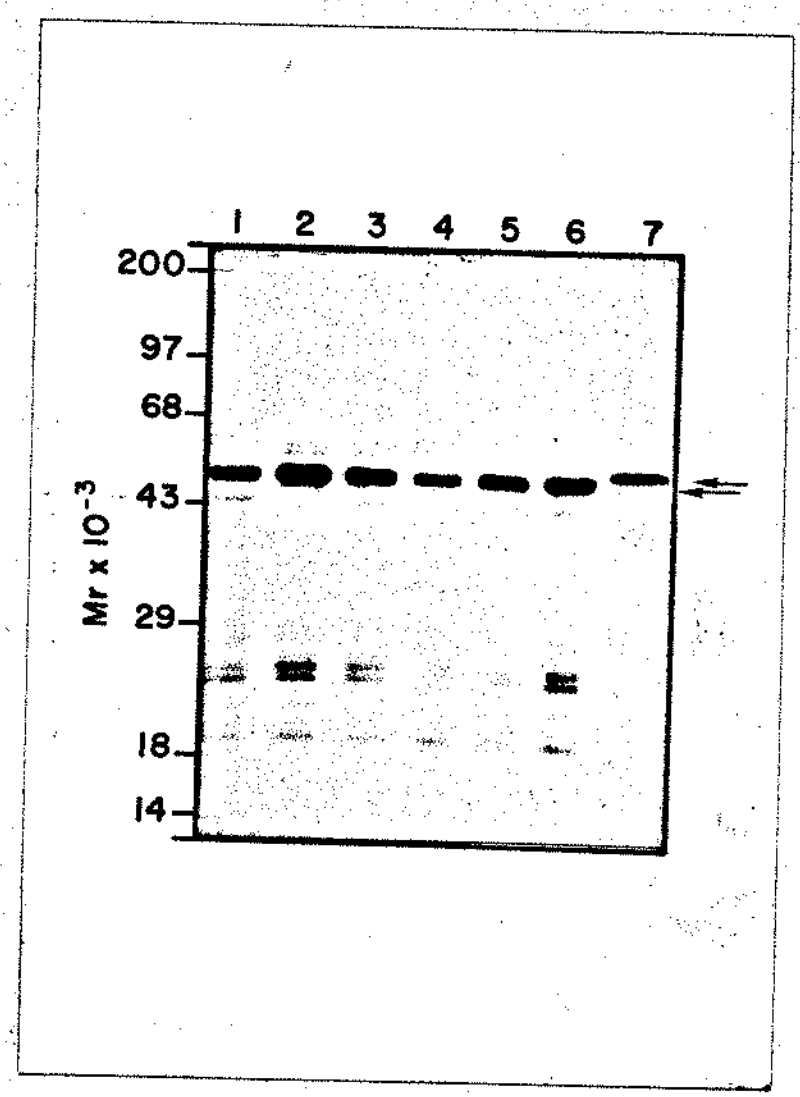
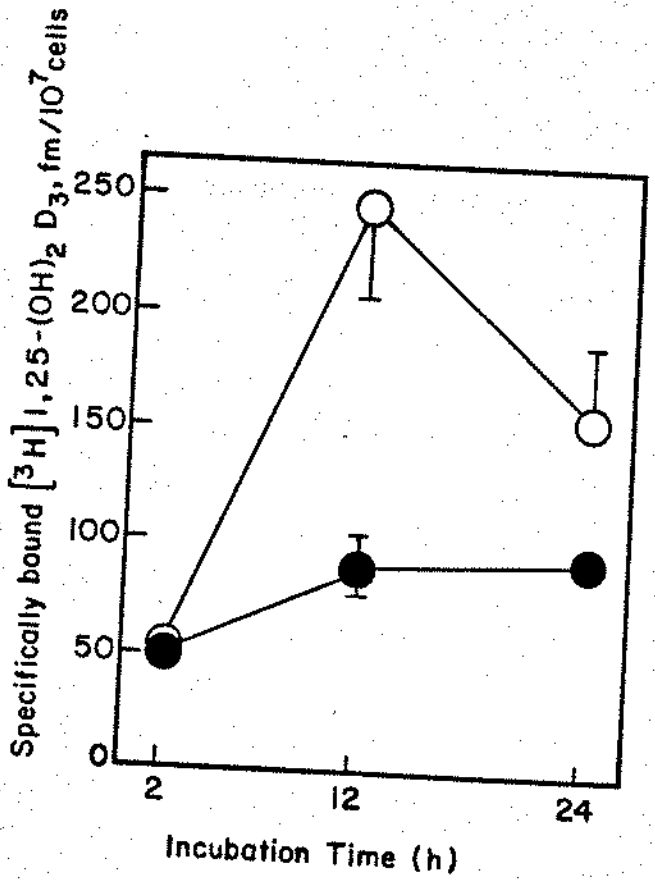

1 2 3 4 5 6 7
200
97
68
43
29
18
14
Mr x 10⁻³

Figure 3. Regulation of [^{3}H]1,25-$(OH)_2D_3$ uptake into intact HL-60 cells by PMA. After cells in SFM were treated with 10 nM PMA (O) or 10 nM 4α-P (●) for 2, 12, and 24 h at 37° C, they were labeled for 2 h with 2 nM [^{3}H]1,25-$(OH)_2D_3$ in the presence or absence of a 200-fold excess nonradioactive hormone. Specifically bound [^{3}H]1,25-$(OH)_2D_3$ in total cells was quantified by the HAP assay. Values represent the mean ± SE from the two separate experiments with four replicate wells.


Specifically bound [3H] 1,25-(OH)2 D3, fm/10^7 cells
250
200
150
100
50
0
2
12
24
Incubation Time (h)

CHAPTER VI

CONCLUDING REMARKS

The receptor proteins for the steroid hormone, 1,25-$(OH)_2D_3$ have been intensively studied mostly in chick intestine (DeLuca and Schnoes, 1983). Receptors for 1,25-$(OH)_2D_3$ have been identified not only in the classical target tissues such as intestine, bone and kidney, but in other normal and neoplastic tissues. 1,25-$(OH)_2D_3$ receptors are also present in hematolymphopoietic tissues including cell lines with monocytic and myeloid characteristics. However, the biological function of 1,25-$(OH)_2D_3$ and the role of its receptor in tumor cell lines remains unknown until quite recently. In 1981, Colston et al., discovered that, besides the well known action of 1,25-$(OH)_2D_3$ in calcium metabolism, 1,25-$(OH)_2D_3$ also suppressed proliferation of malignant melanoma cells. Almost simultaneously, it was reported that 1,25-$(OH)_2D_3$ was capable of inducing differentiation of murine myeloid leukemia cells, M1 cells, (Abe et al., 1981) and human promyelocytic leukemic cells, HL-60 (Miyaura et al., 1981). Subsquently, it was proposed that 1,25-$(OH)_2D_3$ induces differentiation of HL-60 cells by a receptor-mediated mechanism similar to that of 1,25-$(OH)_2D_3$'s classical steroid hormone action (Tanaka et al., 1982; Mangelsdorf et al., 1984).

However, very little is currently known about the physiological significance of this *in vitro* observation or of the mechanism by which it is achieved. Understanding the mechanism by which 1,25-$(OH)_2D_3$ induces HL-60 cell differentiation would shed some light in the molecular events involved in hematolymphopoietic cell maturation. This information also will lend insight into the process whereby diverse biological phenomena are controlled such as osteoclastic mediated bone calcium mobilization as well as into the fundamental mechanisms of cancer.

One of the major problems confronting research using cultured cells has been the variability of results obtained from experiment to experiment on a day-by-day basis. These variabilities may be accounted for, in part, by the variability of the serum in which the cells are grown. Not only does serum contain undefined proteins and factors, but serum can vary widely in its composition from batch to batch.

Therefore, it is necessary to remove undesirable factors present in serum to study the mechanism of action of hormone in the cellular differentiation process (Chapter 2). We have successfully cultured HL-60 cells in serum-free chemically defined medium (SFM), in which cells proliferate indefinitely. 1,25-$(OH)_2D_3$ caused concentration-dependent maturation of HL-60 cells grown not only in 10% SCM but also in SFM, as evidenced by its ability to decrease cell proliferation, and to induce chemiluminescent responsiveness and lysozyme production. Treatment of HL-60 cells with 1,25-$(OH)_2D_3$ resulted in a concentration-dependent increase of α-naphthylacetate esterase enzyme activity which has been shown to occur primarily in monocytes and is virtually absent in granulocytes. In addition, HL-60 cells acquired monocyte-specific cell surface antigens in a dose-dependent manner. These data clearly indicate that HL-60 cells grown in SFM were able to differentiate toward the monocyte/macrophage pathway after exposure to 1,25-$(OH)_2D_3$. Moreover, the data also suggests that 1,25-$(OH)_2D_3$ directly mediates this differentiation process rather than via a factor(s) contained within serum. These results are consistent with the findings of Breitman et al. (1980) who demonstrated that HL-60 cells cultured in serum-free nutrient medium supplemented with transferrin and insulin could differentiate to mature granulocytes in the presence of DMSO.

The doses of 1,25-$(OH)_2D_3$ reported in the literature, which have been shown to induce phenotypic changes in HL-60 cells cultured in 10-20% serum supplemented medium, are generally larger than would be predicted by the equilibrium dissociation constant for its receptor (Tanaka et al., 1982; Mangelsdorf et al., 1984). This incongruence tends to obfuscate the issue as to whether this hormone is important for "normal" hematopoietic maturation. Furthermore, extrapolating these *in vitro* doses to doses that would be clinically effective for treating hematopoietic disorders, raises serious concern, given the potential toxicities of 1,25-$(OH)_2D_3$. We sought to address these issues using a chemically defined medium, not only to assess the dependence of 1,25-$(OH)_2D_3$-induced differentiation on serum, but also to ascertain the potency of vitamin D_3 metabolites under conditions that more closely approximated an extracellular environment. In this regard, 1,25-$(OH)_2D_3$ exhibited a 2- to 20-fold increase in potency for inducing several phenotypic changes in HL-60 cells cultured in SFM. Thus, the phenotypic changes reported by us and others utilizing chemically defined serum-free media occur at concentrations of the active hormone that are more closely associated with physiological concentrations.

The increased potency of 1,25-$(OH)_2D_3$ in SFM or decreased cellular responsiveness in the presence of serum has been demonstrated for a wide variety of cell types (Amento et al., 1984; Ball et al., 1984; Darfler et al., 1980). Thus, the phenotypic changes reported by us and others utilizing chemically defined serum-free media occur at concentrations of the active hormone that are more closely associated with physiological concentrations. The lower potency of 1,25-$(OH)_2D_3$ in HL-60 cells cultured in 10% SCM could be a consequence of the vitamin D-binding protein in serum which could decrease the free hormone concentration in

the culture medium. This view also has been advanced previously by Amento et al. (1984) for 1,25-$(OH)_2D_3$ induced differentiation in U937 cells. In contrast to the uptake of 1,25-$(OH)_2D_3$ in SFM, uptake into intact HL-60 cells is reduced greatly in the presence of 10% fetal calf serum (data not shown). These results are consistent with the hypothesis that serum binding proteins may be acting as a reservoir for the hormone. However, it has been suggested previously that serum binding may not be the only cause for decreases in cell sensitivity to a drug or ligand (Darfler et al., 1980). Therefore, we cannot exclude the possibility that other substances contained in serum could be antagonizing the differentiating effect of 1,25-$(OH)_2D_3$ in HL-60 cells.

The various vitamin D_3 metabolites were examined for their effects on the induction of differentiation of HL-60 cells. The relative order of potency for 1,25-$(OH)_2D_3$ metabolites causing induction of differentiation in HL-60 cells cultured in SFM was 1,25-$(OH)_2D_3$ >> 1-$(OH)D_3$ > 25-$(OH)D_3$ ≅ 24,25-$(OH)_2D_3$. The specificity of these vitamin D_3 metabolites tested in the present study in inducing differentiation was well correlated with the specificity of their association with the putative 1,25-$(OH)_2D_3$ receptor (Tanaka et al., 1982; Ostrem et al., 1987). The existence of a positive correlation between 1,25-$(OH)_2D_3$ induced differentiation and the occurrence of occupied 1,25-$(OH)_2D_3$ receptors has been reported previously in HL-60 cells (Mangelsdorf et al., 1984). Likewise, the functional defect of the 1,25-$(OH)_2D_3$ resistant HL-60 clones appears to be correlated with the reduced amount of the specific 1,25-$(OH)_2D_3$ receptor (Kuribayashi et al., 1983). These results are supportive of the hypothesis that 1,25-$(OH)_2D_3$ induced differentiation in HL-60 cells is receptor mediated, although the mechanism of 1,25-$(OH)_2D_3$-induced differentiation of HL-60 cells remains unknown.

Since 1,25-$(OH)_2D_3$ was shown to be a more potent cellular differentiating agent when cells were cultured in SFM in comparison to SCM, it was important to examine whether the 1,25-$(OH)_2D_3$ receptor had undergone changes in physical/chemical characteristics. Such structural or chemical differences might be related to the observed differences in hormone potency. Similar sedimentation patterns (3.4S) and elution profile of DNA-cellulose chromatography were observed for the [^{3}H]1,25-$(OH)_2D_3$ receptor in HL-60 cells grown in SFM and SCM. The equilibrium dissociation constant of 1,25-$(OH)_2D_3$ receptor and the number of binding sites per cell remained unchanged in HL-60 cells cultured in SFM. Thus, it is unlikey that physical/chemical alterations in 1,25-$(OH)_2D_3$ receptors are responsible for the increased potency of 1,25-$(OH)_2D_3$ in the differentiation of HL-60 cells in SFM.

We established that the HL-60 cell line could proliferate in SFM and was capable of differentiating toward the monocyte/macrophage pathway after exposure to 1,25-$(OH)_2D_3$. Furthermore, culturing HL-60 cells in serum-free conditions does not seem to alter the physical/chemical characteristics of the 1,25-$(OH)_2D_3$ binding protein qualitatively or quantitatively. Many cell types can now be cultured in completely defined media, and the utilization of these systems in the study of molecular mechanism of hormone action may be quite fruitful. Thus further studies can now be pursued utilizing a more "physiological milieu" without the variation introduced by the use of serum.

Since the alteration of receptor level is known to be an important factor in modulating cell responsiveness to hormone, it is essential to know whether the receptor levels are regulated in the target cells. In Chapters 3 and 4, several aspects of 1,25-$(OH)_2D_3$ receptor regulation was investigated using intact HL-60 cells as a

model system since preliminary studies indicated that 1,25-$(OH)_2D_3$ receptors appeared to undergo apparent 'up-regulation' (increase in specifically bound [^{3}H]1,25-$(OH)_2D_3$), followed by apparent 'down-regulation' (loss of specifically bound [^{3}H]1,25-$(OH)_2D_3$) upon continuous exposure to 1,25-$(OH)_2D_3$. An attempt was made to ascertain whether apparent up-regulation and/or down-regulation is due to changes in physical/chemical characteristics in receptors or due to changes in the amount of receptor protein present in cells. The incubation of intact cells with 1-2 nM [^{3}H]1,25-$(OH)_2D_3$ resulted in saturation of 1,25-$(OH)_2D_3$ receptors within 1 to 2 h after hormone addition. Continuous exposure to hormone resulted in a further accumulation of specific 1,25-$(OH)_2D_3$ binding which became maximal between 8 and 16 h. This slower phase of rise in hormone binding appears to be due to an induction event which is presumed to be mediated by nuclear effects. This phase was followed by loss of specific 1,25-$(OH)_2D_3$ binding observed by 24 h after hormone addition. It has been suggested that vitamin D_3 metabolites up-regulated the number of 1,25-$(OH)_2D_3$ receptors by a receptor-mediated induction event in a cultured kidney cell line (Costa et al., 1985). Mutant skin fibroblasts from patients with vitamin D-dependent rickets type II (Hirst et al., 1985), containing nonresponsive 1,25-$(OH)_2D_3$ receptors because of a defect in DNA binding, also failed to exhibit the characteristic up-regulation observed in normal cells (Costa et al., 1985). More recently, it has been demonstrated that 1,25-$(OH)_2D_3$ can increase mRNA activity for its receptor in 3T6 cells, indicative of receptor autoregulation (Mangelsdorf et al., 1987). These experimental results support our observation of homologous up-regulation of 1,25-$(OH)_2D_3$ receptors in a 1,25-$(OH)_2D_3$ target cell.

In order to examine whether up-regulation was associated with secondary binding sites and/or an additional receptor population, we characterized and quantitated up-regulated and down-regulated 1,25-$(OH)_2D_3$ receptors in comparison to basal receptors. Scatchard analysis indicated that only one binding population existed and the apparent equilibrium dissociation constants (K_d's) were not significantly changed in the up- and down-regulated receptors. However, the estimated number of receptor sites was increased 2-fold by 14 h followed by decrease in the number of receptor sites to the basal receptor level by 24 h. Moreover, the data from sucrose gradient analysis, DNA-cellulose chromatography and fast protein liquid chromatography (FPLC) indicated that no significant changes in physical/chemical characteristics occurred with up- and down-regulated receptors. Therefore, our experimental results suggest that 1,25-$(OH)_2D_3$ induces increases followed by decreases in the number of 1,25-$(OH)_2D_3$ receptors in intact HL-60 cells.

To ascertain whether the alterations in receptor level are nuclear associated events, the effect of inhibitors of transcription or protein synthesis on the receptor regulation and the maintenance of basal receptor level was examined using various cytotoxic agents. The finding that the apparent up-regulation of 1,25-$(OH)_2D_3$ receptors in HL-60 cells was dependent on new protein and new mRNA synthesis provides additional evidence that receptor occupancy causes an induction event. The inhibitors of transcription tested lowered the basal receptor level rapidly. Taken together these results suggest that the occupied 1,25-$(OH)_2D_3$ receptor is a dynamic and rapidly turning over protein with a half-life of about 3.5 h. This observation is well correlated with the relatively short half-lives of the estrogen receptor in breast cancer cells and uterine cells (Eckert et al., 1984; Nardulli and

Katzenellenbogen, 1986), the androgen receptors in the ductus deferens tumor cells (Syms et al., 1985) and 1,25-$(OH)_2D_3$ receptor in a cultured kidney cell line (Costa and Feldman, 1987).

The control of the expression of steroid hormone action at the nucleus is not well understood. Some mechanism must exist for the termination of the effect of the hormone-receptor complex. The disappearance of hormone-receptor complexes has been shown to be largely due to a loss of hormone-binding capacity of steroid hormone receptors (processing) (Horwitz and McGuire, 1978a,b). We have shown that intact HL-60 cells exhibited loss of specific 1,25-$(OH)_2D_3$ binding, which can be explained by a decrease in the amount of receptor and/or loss of functional receptor (i.e. processing). Therefore, this rapid loss of specific binding in the intact HL-60 cell may represent the effect of a control mechanism such as receptor processing. The similar alterations in 1,25-$(OH)_2D_3$ receptor level were observed in both human monocytic cell line, U937 and mouse macrophage-like cell line, P388D1 which are also 1,25-$(OH)_2D_3$ target cell lines.

However, conclusions regarding the location and/or quantification of unoccupied or potentially non-hormone binding forms of the receptor cannot be drawn from these ligand binding studies. The availability of monoclonal antibodies to the 1,25-$(OH)_2D_3$ receptor would enable the study of this protein independent of hormone-binding activity. Therefore, immunological techniques were utilized to further identify and detect any changes occurred during the homologous regulation of 1,25-$(OH)_2D_3$ receptor in HL-60 cells (Chapter 4). The monoclonal antibodies used in this study were raised against the porcine intestinal 1,25-$(OH)_2D_3$ receptor, as described previously (Dame et al., 1986). Since the monoclonal antibody IVG recognized all of the 1,25-$(OH)_2D_3$ receptors in the crude nuclear extract of HL-60

cells, immunoprecipitation using IVG was performed to increase the purity and concentration of 1,25-$(OH)_2D_3$ receptor. The enriched 1,25-$(OH)_2D_3$ receptor from HL-60 cells was detected as a single form of 53 kDa using ^{125}I- IVG. The electrophoretic mobilities of the up-regulated and down-regulated 1,25-$(OH)_2D_3$ receptor protein were not altered compared to that of the basal receptor. However, there was a 3-fold increase in the amount of receptor from HL-60 cells treated with 1,25-$(OH)_2D_3$ for 12 h (up-regulated receptor) compared to that from 2 h treatment (basal receptor) as measured by densitometery. This phase was followed by a decrease in the level of 1,25-$(OH)_2D_3$ receptors to the basal level by 36-48 h. The induction of the increase in the amount of 1,25-$(OH)_2D_3$ receptor required new protein synthesis since cycloheximide completely inhibited the rise in the receptor level. Although the immunoblotting assay is not generally sensitive enough for the accurate quantitative determination of receptor protein, these results are well correlated with [^{3}H]1,25-$(OH)_2D_3$ binding studies. Among the plausible methods, RIA may be the best method to quantify 1,25-$(OH)_2D_3$ receptor protein accurately.

While both the immunodetection and ligand binding methods estimated a similar increase in the amount of 1,25-$(OH)_2D_3$ receptor induction during up-regulation, they differed quantitatively for estimating the degree of down-regulation. Utilizing Western blotting, the level of the up-regulated receptor was observed to decline to basal levels by 36-48 h and remained at that level up to 72 h during continuous hormone incubation. Conversely, [^{3}H]1,25-$(OH)_2D_3$ binding data showed that at 48 h and 72 h, up-regulated receptors had declined to 46% and 66% of the basal level, respectively. This difference may be interpreted to mean that although receptor protein is present during down-regulation as detected by Western blots, the loss of specific [^{3}H]1,25-$(OH)_2D_3$ binding indicates that the

ligand binding site is lost preferentially or at least incapable of binding ligand. Similarly, it has been reported that the level of 1,25-$(OH)_2D_3$ receptors in human breast cancer cells undergoes homologous down-regulation by continuous hormone exposure (Sher et al., 1985).

Therefore, the down-regulation of the 1,25-$(OH)_2D_3$ receptor observed in HL-60 cells seemes to be due to partly a decrease in the amount of receptor protein and partly a loss of functional receptor (processing). However, Radparvar and Mellon (1986) have demonstrated that recycling of receptor played an important functional role for the replenishment of unoccupied 1,25-$(OH)_2D_3$ intestinal receptors in chick and cycloheximide had no effect on the time-course or the magnitude of replenishment of nuclear receptor. Similarly, the estrogen receptor replenishment in the rat uterus was entirely due to receptor recycling, not due to receptor processing or synthesis of new receptors (Kasis and Gorski, 1981). Since these studies were based on the hormone binding activity of the receptor without measuring the amount of receptor protein present in the target tissues, non-functional forms of receptors could not be detected. However, it cannot be excluded that a decreased recycling of the receptor might be involved in the down-regulation of 1,25-$(OH)_2D_3$ receptor in HL-60 cells.

We have demonstrated for the first time the up-regulation of 1,25-$(OH)_2D_3$ receptor followed by down-regulation in intact human target cells cultured in serum-free condition. Maintenance and apparent up-regulation of receptor require new mRNA and protein synthesis. The 1,25-$(OH)_2D_3$ receptors in HL-60 cells are a single form of protein species of 53 kDa and the pig intestinal receptors are detected at 55 kDa as described previously (Dame et al., 1985). These results are consistent with the conclusions that 1,25-$(OH)_2D_3$ receptors are protein species ranging from

52 to 60 kDa and though their functional and immunological domains have been evolutionarily conserved, an inverse relationship apparently exists between phylogenic status and receptor mass (Mangelsdorf et al., 1987). The data presented here suggest that the homologous up-regulation followed by down-regulation of 1,25-$(OH)_2D_3$ receptor is due to changes in the amount of receptor protein and may represent a nuclear mechanism for the control of cellular responsiveness to hormone in the intact human target cells.

The alterations of the amount of receptor protein in HL-60 cells might be due to increases or decreases in the rates of receptor synthesis and/or degradation. A 'density shift technique' could be utilized to measure the changes in the true rates of receptor synthesis and degradation. The density labeling of receptor would provide a clear basis for distinguishing between old receptor and newly synthesized receptor without the need for extensive purification of receptor or the use of inhibitors of protein or mRNA synthesis. This method has been used to determine rates of synthesis and degradation of acetylcholine (Gardner and Fambrough, 1979), insulin (Reed and Lane, 1980), glucocorticoids (Raaka and Samuels, 1983), and estrogen (Eckert et al., 1984) receptors, and should be applicable to any receptors. The physiological significance of our findings that HL-60 cells may regulate 1,25-$(OH)_2D_3$ receptors as they differentiate into monocytes remains unknown. This may reflect a portion of an internal mechanism to enhance or change responses to an endogenous ligand for its receptor as cells mature.

Since 1,25-$(OH)_2D_3$ is the most potent inducing agent for monocytic differentiation of HL-60 cells, the possibility that these alterations might be components of the cellular differentiation process was investigated in Chapter 5. Cells were treated with monocytic inducers or granulocytic inducers of HL-60 cells

for up to 48 h. At the indicated time, cells were harvested to measure the specific [^{3}H]1,25-$(OH)_2D_3$ binding in intact cells. 10 nM phorbol 12-mirystate 13-acetate (PMA), a monocytic inducer, increased the specific [^{3}H]1,25-$(OH)_2D_3$ binding observed by 12 h. This phase was followed by a decrease in the specific [^{3}H]1,25-$(OH)_2D_3$ binding observed by 24-48 h. The regulation of [^{3}H]1,25-$(OH)_2D_3$ binding by PMA was in a similar manner to that by 1,25-$(OH)_2D_3$.

Like 1,25-$(OH)_2D_3$, phorbol esters induce HL-60 cell differentiation at low concentrations and they have an integral effect on cell growth and proliferation (Rovera et al., 1979; Vandenbark and Neidel, 1984). The monocyte-macrophage phenotype induced by 1,25-$(OH)_2D_3$ in the HL-60 cells was found to be similar but not identical to that induced by PMA (Murao et al., 1983). Protein kinase C (the Ca^{++}/phospholipid-dependent enzyme) is a phospholipid-sensitive Ca^{++}-dependent kinase that is the proposed receptor for tumor-promoting phorbol esters (Castagna et al., 1982; Kaibuchi et al., 1983). Since 1,25-$(OH)_2D_3$ modulates cell Ca^{++} content and phosphatidylserine synthesis (Simpson and Arnold, 1986; Matsumoto et al., 1985; Miyaura et al., 1984), agents that are involved in protein kinase C activation, and since phorbol esters and 1,25-$(OH)_2D_3$ induce differentiation, it seems likely that the mechanisms of differentiation between 1,25-$(OH)_2D_3$ and phorbol esters are related. The cells treated with retinoic acid or dimethyl sulfoxide, known granulocytic inducers of HL-60 cells, did not exhibit the regulation of specific [^{3}H]1,25-$(OH)_2D_3$ binding in HL-60 cells. These data suggest that the regulation of 1,25-$(OH)_2D_3$ receptor in HL-60 cells might be associated with the monocytic differentiation process.

The regulation of specific [^{3}H]1,25-$(OH)_2D_3$ uptake into HL-60 cells by PMA was examined using immunological techniques. The monoclonal antibody,

IVG8C11, raised against the 1,25-$(OH)_2D_3$ receptor in porcine intestines (Dame et al., 1984) was utilized for the immunoprecipitation and immunoblot assay. The HL-60 cells in SFM were treated with 10 nM PMA or 10 nM 4α-phorbol-12,13-didecanoate (4α-P), an analogue incapable of stimulating protein kinase C and therefore presumed to be biologically inactive (Hecker, 1971), to ascertain the specific effect of PMA on the 1,25-$(OH)_2D_3$ receptor regulation. After the immunoprecipitated receptors were electrophoresed on SDS-polyacrylamide gel, the proteins on gel were transferred to polyvinylidene difluoride (PVDF) membranes which were subsequently probed with ^{125}I-IVG8C11. The 1,25-$(OH)_2D_3$ receptor was detected as a single form of 53 kDa protein calculated from this and other gels and its electrophoretic mobility was not altered by PMA-treatment (Chapter 5). However, the amount of 1,25-$(OH)_2D_3$ receptor detected by ^{125}I-IVG8C11 was increased by 12 h as compared to those with 2 h-treatment and then decreased slightly by 24 h (lane 1 to 3). The level of receptors in the cells treated with 4α-P for 12 and 24 h seemed to be increased as compared to basal level. The ratios of densities of the bands are 1.0: 2.5: 1.7: 0.5: 1.0: 1.6 for the cells treated with PMA for 2, 12, and 24 h and with 4α-P for 2, 12, and 24 h, respectively as measured by a Soft Laser scanning densitometer.

The specific [^{3}H]1,25-$(OH)_2D_3$ binding activity for cells treated with PMA was compared to those treated with 4α-P. The specific [^{3}H]1,25-$(OH)_2D_3$ was increased by 12 h incubation with PMA followed by the decrease in [^{3}H]1,25-$(OH)_2D_3$ binding by 24 h. The specific [^{3}H]1,25-$(OH)_2D_3$ binding was also increased by the treatment with 4α-P as compared to the basal level. These data correlate well with the results of immunoblotting assays although the immunoblotting assay is not generally sensitive for the accurate quantification of the

protein. The phenotypic changes associated with differentiation were examined for 4α-P. The nitroblue tetrazolium (NBT) reduction assay, which measures the ability of cells for the production of superoxide, indicated that 4α-P did not induce the differentiation of HL-60 cells. In addition, cells treated with 4α-P did not adhere to the bottom of flasks, while cells with PMA mostly adhered to the flasks (data not shown). Cells were induced to differentiate by incubation with 10 nM 1,25-$(OH)_2D_3$ for 3 days, indicating a normal differentiational response of HL-60 cells cultured in SFM to inducing agents.

This study attempts to provide insight into the mechanism of the monocytic differentiation of HL-60 cells by examining the regulation of 1,25-$(OH)_2D_3$ receptor. The data presented here suggest that the regulations of 1,25-$(OH)_2D_3$ receptors in HL-60 cells might be components of the cellular differentiation process induced by monocytic inducers. The homologous or heterologous alterations of 1,25-$(OH)_2D_3$ receptor number in target cells are found under many conditions. HL-60 cells have shown to regulate other types of receptors during monocytic and granulocytic differentiation, including receptors for insulin and phorbol diester (Chaplinski et al., 1986; Martell et al., 1987; Lane et al., 1986). The regulation of hormone receptors in association with functional differentiation also has been reported in other cell systems (Mezzetti et al., 1987; Anselmet et al., 1984). However, the reason or mechanism for this change in the amount of 1,25-$(OH)_2D_3$ receptor in HL-60 cells is not apparent.

Most differentiation-associated phenotypic changes in HL-60 cells appeared after at least 24 hour-treatment with 1,25-$(OH)_2D_3$, while the up-regulation of 1,25-$(OH)_2D_3$ receptors occurred between 3-12 h after exposure to 1,25-$(OH)_2D_3$. The phenotypic changes are preceded by changes in the expression of several

oncogenes. A rapid decrease in the expression of c-myc and c-myb with 50% reduction has been demonstrated in HL-60 cells treated with 1,25-$(OH)_2D_3$ or PMA (Reitsma et al., 1983; Westin et al., 1982). An induction of c-fos gene with maximal levels in 20-30 min and a rapid induction of c-fms as well as the increased level of $pp^{60\ c\text{-}src}$ protein has been reported in PMA and 1,25-$(OH)_2D_3$ treated HL-60 cells (Mitchell et al., 1985; Brelvi et al., 1986; Gee et al., 1986). The regulation of expression of cellular oncogenes has been considered to be linked to the malignancy and the differentiation of cells. Therefore, it could be speculated that the changes in the level of hormone receptor may be important for the early cellular changes which are fundamental to the differentiation, rather than the consequences of the cellular differentiation. In addition, a commitment to increased insulin receptor expression was demonstrated after 1 h exposure to 1 μM 1,25-$(OH)_2D_3$ in HL-60 cells. A significant increase in phorbol ester binding was observed 12 h after addition of 1,25-$(OH)_2D_3$ to HL-60 cells (Martell et al., 1987) and incubation of the HL-60 cells with PMA induced cell attachment starting before 12 h (Lotem and Sachs, 1979).

Therefore, an increase in 1,25-$(OH)_2D_3$ receptor binding may serve as an early and sensitive marker of monocytic differentiation in HL-60 cells. It could be speculated from the present study and others that the increase in the number of 1,25-$(OH)_2D_3$ receptor augments the cell's sensitivity to external hormonal stimuli, which may be required for the monocytic differentiation but not for the granulocytic differentiation. The loss of specific hormone binding may represent a control mechanism for the termination of the effect of the increased hormone-receptor complex. Further studies concerning the biochemical basis for 1,25-$(OH)_2D_3$

receptor regulation should lead to new understanding of the possible role of 1,25-$(OH)_2D_3$ receptors in the differentiation process and receptor dynamics.

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APPENDIX

INDUCTION OF MONOCYTIC DIFFERENTIATION BY CALCITRIOL (1,25-DIHYDROXYVITAMIN D_3) IN THE HUMAN PROMYELOCYTIC LEUKEMIC CELL LINE (HL-60) IN SERUM-FREE MEDIUM

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ABSTRACT

The effect of calcitriol on the induction of differentiation in human promyelocytic leukemic cell line (HL-60) cultured in serum free chemically defined medium (SFM) was investigated. The utilization of SFM containing RPMI-1640 basal medium supplemented with insulin (5 mg/ml), transferrin (5 mg/ml), sodium selenite (5 ng/ml), and bovine serum albumin (0.5 mg/ml), allowed a more precise examination of the cellular/molecular mechanism of calcitriol's action in HL-60 cell differentiation without interference of components present in serum. HL-60 cells grown in SFM were induced to differentiate into monocytes/macrophages by calcitriol as indicated by induction of differentiation associated biological and biochemical parameters, chemiluminescent (CL) responsiveness, lysozyme activity, nonspecific esterase, expression of cell surface antigens and reduced proliferation. The exposure of HL-60 cells in SFM to calcitriol (from 10^{-10} to 10^{-8} M) resulted in dose-dependent induction of these parameters, which is similar to those obtained with cells grown in 10% fetal calf serum containing medium (10% SCM). However, calcitriol was 5-fold more potent for HL-60 cells cultured in SFM than those in 10% SCM as indicated by shifts in dose-response curves for induction of CL responsiveness and lysozyme activity. The effect of calcitriol on the proliferation and acquisition of several monocyte-associated cell surface antigens was also more sensitive for HL-60 cells cultured in SFM than those in cells grown in 10% SCM. We characterized and quantitated calcitriol receptors in HL-60 cells cultured in SFM in comparison to those in 10% SCM after exposing intact cells to radio-labeled calcitriol. Cells cultured in either SFM or 10% SCM exhibited calcitriol receptors that migrated at 3.4S as a single peak on sucrose gradients and elicited inherent DNA binding ability. There was essentially no difference in the

apparent dissociation constants (K_d's) nor in the number of calcitriol binding sites per HL-60 cell, that is 6.0×10^{-11} M and 3,000 binding sites/cell, respectively. It is concluded that culturing HL-60 cells in SFM results in full expression of calcitriol induced phenotypic changes excluding the possibility that such changes result from the indirect effect of calcitriol mediated by identified and/or unidentified components present in serum.

INTRODUCTION

The biologically active metabolite of calciol, 1,25-dihydroxyvitamin D_3 (calcitriol) has been shown to play a major role in mineral metabolism at such target tissues as intestine, bone and kidney (see [1] and [2] for reviews). The mode of calcitriol's action within target tissues appears to be similar to that of steroid hormones [3], in that calcitriol first binds to a specific, high affinity receptor which consequently induces the expression of mineral-regulating proteins [4-6]. Receptors for calcitriol have been identified not only in the "classical target tissues" [7-9], but in other normal [10,11] and neoplastic tissues [12-14] including cells of the hematolymphopoietic tissues [15-18]. Furthermore, calcitriol receptors are present in cell lines with monocytic and myeloid characteristics [15-18]. In these neoplastic and monocytic/myeloid calcitriol receptor positive cells, calcitriol has several actions. Calcitriol has been shown to suppress the proliferation of malignant melanoma cells [19]. Moreover, calcitriol promotes monocytic-like differentiation in murine myeloid leukemia cells (M1) [20], human promyelocytic leukemia cells (HL-60) [21-23] and monoblastic U937 cells [24]. These findings together with the demonstration that calcitriol mediates the differentiation of HL-60 cells into active bone resorbing macrophages [25] engenders the distinct possibility that calcitriol may play a central role in the maturational processing of hematolymphopoietic, immune and bone resorbing cells.

The availability of several clonal calcitriol receptor positive cell lines should enhance our ability to ascertain calcitriol's role in cellular differentiation. In particular, HL-60 cells may provide a suitable model system for these investigations, especially for describing the involvement and regulation of the calcitriol receptor in the cellular differentiation process. Among various inducers

such as vitamins, tumor promoters, highly polar compounds, and anticancer agents [26-30], calcitriol has been shown to be the most potent inducer of HL-60 differentiation [21]. The potency of several calciol analogs has been correlated roughly with their specificity and affinity for the calcitriol cytosolic receptor in HL-60 cells [15]. However, due to the presence of serum in the culture medium during these studies, absolute potencies for calciol metabolite could not be assessed accurately. While the evidence to date suggests that calcitriol directly mediates HL-60 cellular differentiation, it is conceivable that components contained within the serum-supplemented medium may be responsible, in part, for modifying the calcitriol response. Aside from the known components contained in serum that could induce or modify HL-60 differentiation (i.e., retinoids, interferons, calciol metabolites and other hormones), growth factors and other unknown components may obfuscate calcitriol's mode of action [31,32]. Serum also contains a number of proteins, including vitamin D-binding proteins which could alter calcitriol pharmacokinetics. In addition, serum can vary widely in its composition from batch to batch. Thus the interpretation of the experimental results can be complicated not only by the presence of serum, but by the variation in the makeup of its components. Therefore, elimination of undefined substances, hormones and differentiating factors present in serum from the culture medium should facilitate studies assessing calcitriol's action on growth and differentiation of these human promyelocytic leukemia cells.

In the present study, we report that HL-60 cells proliferate continuously in a serum-free chemically defined medium (SFM) which is similar in composition to one described by Breitman et al. [33]. Moreover, cells grown in SFM retain the capacity to differentiate to mature monocytes when incubated with calcitriol.

Lastly, analysis of occupied calcitriol receptors in HL-60 cells cultured in SFM and serum-containing medium (SCM) indicates that serum-free conditions do not alter the physical/chemical properties of occupied calcitriol receptors.

MATERIALS AND METHODS

Materials. Calcitriol, radiolabeled with tritium at the 26,27-methyl groups ([^{3}H]calcitriol, 180 Ci/mmol), was obtained from the Radiochemical Center (Amersharm, Buckshire, UK). Nonradioactive calcitriol, (24R)-hydroxycalcidiol, and (1S)-hydroxycalciol were gifts from Dr. M. Uskokovic of Hoffmann-LaRoche, Inc. (Nutley, NJ), and calcidiol from Dr. Paul W. O'Connell of the Upjohn company (Kalamazoo, MI). Phorbol 12-myristate-13-acetate (PMA) was purchased from Sigma (St. Louis, MO), hydroxylapatite from Bio Rad Laboratories (Richmond, CA), and Dulbecco's phosphate buffered saline (PBS) from GIBCO (Grand Island, NY). Dimethylsulfoxide (DMSO) was purchased from Fisher Scientific Company (Fair Lawn, NJ), and 3-amino-phthal-hydrazide (luminol) from Aldrich Chemical Company, Inc. (Milwaukee, WI). All other reagents were of analytical grade.

Buffers. The buffers used throughout these experiments included: 50 mM Tris-HCl, pH 7.5, 1.5 mM EDTA, 2 mM dithiothreitol (DTT) (TED); 50 mM Tris-HCl, pH 7.5, 1.5 mM EDTA, 5 mM DTT, 10 mM sodium molybdate (TEDM); TEDM with various concentrations of KCl (e.g., TEDMK-0.03 = 0.03 M KCl in TEDM); 10 mM Tris-HCl, pH 7.5, 0.5 mM DTT, 0.03 M KCl (TDK); 10 mM Tris-HCl, pH 7.5, 0.5 mM DTT, 0.1% Triton X-100 (v/v) (TDT).

Cell Culture. The human promyelocytic leukemia cells, HL-60, isolated from the peripheral blood leukocytes of a patient with acute promyelocytic leukemia [34] were obtained from American Type Tissue Culture (Bethesda, MD) and were maintained in RPMI-1640 medium buffered with 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) (Sigma Chemical Co., St. Louis, MO) containing 100 units/ml penicillin and 0.5 mg/ml fungizone. This basal medium was supplemented with either heat inactivated (56° C for 30 minutes) 10% (v/v) fetal calf serum (Sterile System, Inc., Logan, UT), designated as 10% SCM, or with a defined supplement of insulin from bovine pancreas (5 mg/ml), human transferrin (5 mg/ml), sodium selenite (5 ng/ml), and bovine serum albumin (BSA) (fatty acid free) (0.5 mg/ml) (Sigma Chemical Co., St. Louis, MO), designated as serum free medium (SFM). Cells were grown at 37° C in a humidified 95% air-5% CO_2 atmosphere and subcultured with 4-5 day intervals at a seeding density of 2×10^5 cells per ml. Cells subcultured in SFM were maintained in this defined nutrient medium for several weeks involving at least 5 to 10 passages before experiments were initiated. Cell proliferation was assessed by counting cells in a hemocytometer, and cell viability was determined by trypan blue dye exclusion.

Calciol Metabolite Treatment. HL-60 cells were seeded in multiwell plates with either 10% SCM or SFM. Except where indicated, cells were treated for 3 days in duplicate wells with various concentrations of calciol metabolites, calcitriol, (24R)-hydroxycalcidiol, (1S)-hydroxycalciol and calcidiol, dissolved in ethanol. Control cultures were incubated with an appropriate amount of vehicle (ethanol). The final ethanol concentration was less than 0.1% (v/v).

Chemiluminescence Assay. Determination of chemiluminescent (CL) responsiveness was carried out as described elsewhere [35,36]. Briefly, calciol metabolite treated HL-60 cells and control cells (10^6) were added to 7.0 ml-glass scintillation vials. After cells were washed once with 2.0 ml of DPBS and once with 1.0 ml of Krebs Ringer phosphate buffer, pH 7.4, containing 0.2% BSA and 0.2% glucose (KRP), they were resuspended in 1.0 ml of KRP buffer followed by the addition of 10 ml of luminol and 10 ml of PMA at a final concentration of 10^{-8} M and 10 mg/ml, respectively. CL measurements were made serially in a Packard Tri-Carb ambient-temperature liquid scintillation counter with use of the tritium window setting and with the coincident circuit turned off.

Lysozyme Activity. Lysozyme activity was determined as described elsewhere [37], with a slight modification. Briefly, the HL-60 cells (1×10^6) were centrifuged at 500 g for 5 min to obtain the supernatant after treatment with various calciol metabolites. The enzyme reaction was initiated by adding 0.1 ml aliquots of supernatant to 0.9 ml of 0.066 M phosphate buffer, pH 6.24, containing 0.15 mg of dry *Micrococcus lysodiekticus* (Sigma, St. Louis, MO) at 30° C. The turbidity of the reaction mixture was measured by spectrophotometry at 450 nm for 20 min.

Nonspecific Esterase Activity. Cytochemical assays for nonspecific esterase activity (α-naphthylacetate esterase) were performed with a commercially available kit (Sigma technical bulletin, No. 90). The enzyme activity can be detected by the formation of highly colored deposits at the sites of enzyme activity. The results are expressed as the percentage of cells with black granulation regardless of the intensity of the coloration. A field of at least 200 cells was counted in a hemocytometer for each determination.

Indirect Immunofluorescence. To measure the binding to cells of monoclonal antibodies (Ab's) which react primarily with monocytes and macrophages, indirect immunofluorescence assays were performed using a fluorescence-activated cell sorter (FACS IV; Becton Dickinson, Mountain View, CA) as described by the manufacturer's direction (Coulter Immunology, Hialeah, FL). Briefly, HL-60 cells (1×10^6) were placed into 12×75 mm Falcon tubes and washed with ice-cold DPBS two times. The reaction of monoclonal Ab with cells were initiated by adding 200 ml of monoclonal Ab solutions, Anti-Leu-M3 (Becton Dickinson Monoclonal Center, Inc., Mountain View, CA) or 63D3 (Bethesda Research Laboratories, Inc., Gaithersburg, MD) to cells and incubated at 4° C for 30 min. After washing two times with 1 ml of wash media (PBS containing 2% (v/v) fetal calf serum and 0.01% (w/v) sodium azide), 200 ml of fluorescein isothiocyanate conjugated second Ab (goat antiserum to mouse immunoglobulin, Coulter Immunology) was added to the cell pellet and incubated at 4° C for 30 min. After the third wash with resuspension media (PBS containing 0.01% (w/v) sodium azide), cells were then resuspended in 1 ml of wash media and subjected to FACS IV analysis. The negative control (only second Ab was added to the control cells) was employed for the determination of background.

Preparation of Calcitriol Receptors. HL-60 cells (50×10^6) cultured in either 10% SCM or in SFM were rinsed and resuspended in 2 ml of serum-free RPMI-1640 medium and SFM, respectively. After cells were labeled with 1 nM [^{3}H]-calcitriol in the presence or absence of 200-fold excess of calcitriol for 1.5 hour at 37° C in a humidified 95% air-5% CO_2 atmosphere, cells were rinsed two times in DPBS followed by centrifugation at 500 g for 5 min. The following manipulations were carried out between 0° C and 4° C. After cells were

resuspended and incubated for 20 min in 2 ml of ice-cold TED buffer, cells were homogenized using a Tissuemizer type SDT (Tekmar Co., Cincinnati, OH) for 3 sec at a speed setting of 50. The homogenate was centrifuged for 10 min at 800 g and the pellet was designated as a crude nuclear fraction. The crude nuclear pellet was incubated with TEDMK-0.3 for 30 min with intermittent vortexing followed by centrifugation at 105,000 g for 45 min in a Beckman L5-50B ultracentrifuge using a type Ti-50 rotor (Beckman Instruments, Palo Alto, CA). The supernatant was utilized as the source of [^{3}H]calcitriol labeled nuclear receptor for physical/chemical characterization.

Sucrose Density Gradient Analysis. Aliquots of [^{3}H]calcitriol receptor were applied to linear sucrose density gradients (4-20%) in TEDMK-0.3 to observe sedimentation characteristics of calcitriol-receptor complexes, as described elsewhere [38]. Gradients were centrifuged for 18 hr at 235,000 g in a Beckman ultracentrifuge (model L5-50B) using a SW-60 rotor between 0-4° C and subsequently fractionated as described elsewhere [38]. To each 0.1 ml fraction was added 4.0 ml of a scintillation fluid [39] and the radioactivity was determined by liquid scintillation spectrometry in a Packard Tri-Carb Spectrometer (model Prias PLD) with an efficiency for tritium of 40%. Quench correction was determined by the use of automatic external standardization.

Binding Studies. HL-60 cells grown in either 10% SCM or in SFM were rinsed and resuspended at a density of 10×10^6 cells per 1 ml of serum-free RPMI-1640 medium and SFM, respectively. After HL-60 cells were labeled with various concentrations of [^{3}H]calcitriol in the presence or absence of a 200-fold excess of calcitriol for 1 hour at 37° C in a humidified 95% air-5% CO_2

atmosphere, cells were rinsed two times with DPBS and resuspended in 1 ml of ice-cold TED for 20 min with intermittent vortexing. Receptor-bound [^{3}H]calcitriol was quantitated by an hydroxylapatite (HAP) batch assay [40] with slight modification. Briefly, 0.3 ml of HAP slurry (50% v/v in TDK buffer) was added to each incubation tube containing 0.3 ml of [^{3}H]calcitriol labeled receptor. The samples were kept at 0-4° C for 15 min with intermittent mixing and centrifuged at 1,500 g for 5 min. The HAP pellet was washed three times with 1.0 ml of TDT buffer, extracted twice with 1.0 ml portions of chloroformmethanol (1:2, v/v), the supernatants combined and dried under a stream of air at 35° C. To each vial was added 4.0 ml of a scintillation fluid [39] and the radioactivity was quantitated by liquid scintillation spectrometry with an efficiency for tritium of 45%.

DNA-Cellulose Chromatography. DNA-cellulose chromato-graphy was performedas described elsewhere [41]. Briefly, the [^{3}H]calcitriol receptor was treated with dextran-coated charcoal to remove unbound calcitriol. The labeled nuclear receptor was diluted ten times (v/v) with TEDM to reduce ionic strength (i.e., to a final concentration of KCl equal to 30 mM) and aliquots were applied to a 10 ml DNA-cellulose column previously equilibrated with TEDMK-0.03 buffer. Chromatography was initiated by washing the column with TEDMK-0.03 buffer, followed by a linear KCl gradient (TEDMK-0.03-0.50) for the elution of labeled receptor. Fractions (2.5 ml) were collected and 1.5 ml aliquots were removed to determine the radioactivity as described above.

Statistical Analysis. Where appropriate, statistical comparisons of the independent samples means were made using Student's t-tests.

RESULTS

Effect of Calcitriol on HL-60 Cell Proliferation. In SFM, growth of HL-60 cells continued at a rate approximately 73% of that occurring in 10% SCM (Fig. 1). Subsequently, we have observed more similar growth rates (i.e., a range of 80-90% of that occurring in 10% SCM) when cells are seeded in SFM at a density of 4-5 $\times$ 10^5 cells/ml. Treatment with calcitriol suppressed proliferation of HL-60 cells in a dose- and time-dependent manner. Figure 1 shows a biphasic dose response in which a slight stimulation of growth occurred in cells cultured in SFM with 0.2 nM calcitriol. At a concentration of 10 nM calcitriol, the viable cell number was reduced to 75% and 50% of control on day 4 in 10% SCM and SFM, respectively.

Chemiluminescent Responsiveness. Chemiluminescence may be viewed as a manifestation of the microbicidal activity of monocytes and granulocytes. To assess the CL responses of HL-60 cells, cells were treated with various concentrations of calciol metabolites for three days (preliminary studies indicated that 3-day treatment was the optimum for determination of CL responses). The results in Figure 2 demonstrate the dose-response relationship for several calciol metabolites and the induction of CL in cells cultured in 10% SCM and SFM. The dose of calcitriol which induced 50% of maximal CL was estimated to be 1.6 nM and 7.8 nM in HL-60 cells grown in SFM and 10% SCM, respectively. The maximal level of CL response induced by calcitriol in cells grown in SFM was not significantly different from that in 10% SCM ($P > 0.05$). Next, effects of other metabolites, (1S)-hydroxycalciol, (24R)-hydroxycalcidiol and calcidiol on the induction of CL were compared in SFM and 10% SCM. Of these metabolites

tested, only (1S)-hydroxycalciol was capable of inducing significantly higher CL in HL-60 cells, albeit requiring doses of several orders of magnitude greater than for calcitriol.

Lysozyme Production. Normal macrophage can synthesize and secrete in vitro copious amounts of lysozyme which is one of the antibacterial proteins [42], and calcitriol treated HL-60 cells exhibit much the same characteristics. The dose-response effects of various calciol metabolites on the production of lysozyme are shown in Figure 3. Preliminary data indicated that 3-day treatment with calcitriol was optimum to measure lysozyme activity. The dose of calcitriol which induced 50% maximal lysozyme production was estimated to be 0.9 nM and 4.4 nM in HL-60 cells grown in SFM and 10% SCM, respectively. The maximal lysozyme induction by calcitriol in cells grown in SFM was not significantly different from that in 10% SCM ($P > 0.05$). The order of the potency in inducing lysozyme activity was similar to that in inducing CL. These results, together with those in Figure 2, indicate that the potency of calciol metabolites in inducing differentiation were calcitriol >> (1S)-hydroxycalciol > calcidiol ≅ (24R)-hydroxycalcidiol in cells grown in SFM.

Nonspecific Esterase Activity. α-Naphthylacetate esterase is detected primarily in monocytes and is virtually absent in granulocytes. Untreated HL-60 cells exhibited little enzyme activity whereas treated HL-60 cells in either SFM or 10% SCM became a-naphthylacetate esterase positive in a dose-dependent manner, with over 50% of cells stained at a dose of 10 nM calcitriol (Table 1). Albeit, it was apparent by visual examination that the intensity of the color for cells treated with

calcitriol and cultured in SFM was greater than for those in 10% SCM (data not shown).

Indirect Immunofluorescence. To detect cell surface specific antigen expression characteristic of mature monocytes and macrophages, indirect immunofluorescence was performed using monoclonal Ab. While untreated HL-60 cells have little reactivity with two monoclonal Ab's tested, monocyte-specific cell surface antigens clearly were detected on calcitriol treated cells in a dose-response manner (Table 2). Between 60% and 80% of HL-60 cells grown in 10% SCM and SFC, respectively, express both Leu-M3 and 63D3 antigens with 20 nM calcitriol treatment. In addition, data in Table 2 reveal that calcitriol exhibits a greater potency for stimulating both Leu-M3 and 63D3 antigens in cells cultured in SFM.

Characterization and Quantitation of Calcitriol Receptor. For the investigation of the mechanism whereby calcitriol induces differentiation in HL-60 cells, a series of experiments for the characterization of calcitriol specific receptor were performed. Since we attempted to remove the unknown factors in serum by using SFM, it was necessary to characterize and quantitate calcitriol receptors in HL-60 cells cultured in SFM and 10% SCM.

The specific uptake of [^{3}H]calcitriol into intact HL-60 cells was measured as described in Materials and Methods section. Fig. 4 demonstrates the saturable and high affinity specific binding of [^{3}H]calcitriol for intact HL-60 cells. Examination of the specific binding by Scatchard analysis reveals linear plots whose apparent equilibrium dissociation constants are 5.7×10^{-11} M and 6.4×10^{-11} M for HL-60 cells cultured in 10% SCM and SFM, respectively. An extrapolation to the abscissa is estimated to be ~3,000 calcitriol binding sites per HL-60 cell either cultured in

10% SCM or in SFM. Aliquots of [^{3}H] calcitriol receptors, prepared by labeling intact HL-60 cells cultured in 10% SCM or SFM with [^{3}H] calcitriol (see Materials and Methods section) were analyzed by sucrose density gradient centrifugation (data not shown). A peak with sedimentation coefficient of 3.4S was found for HL-60 cells cultured in either SFM or 10% SCM. In both cases the peak was completely displaced in the presence of a 200-fold excess of unlabeled calcitriol. To demonstrate and compare the affinity of the calcitriol-receptor complex for DNA, the calcitriol receptor from HL-60 cells cultured in either SFM or 10% SCM was applied to a DNA-cellulose column and eluted with a buffered salt gradient, 0.03-0.5 M KCl (data not shown). One peak of [^{3}H] calcitriol binding component in HL-60 cells cultured in either 10% SCM or SFM appears, which is retained under low ionic strength and elutes at a KCl concentration of 0.18 M.

DISCUSSION

One of the major problems confronting research using cultured cells has been the variability of results obtained from experiment to experiment on a day-by-day basis. It has been reported that 26% of HL-60 cells cultured in 10% fetal calf serum containing medium became phagocytic cells after exposure to 10 nM calcitriol for three days [18], while over 50% of HL-60 cells become phagocytic cells under the same culture condition in another laboratory [21]. It has been also shown that 54% of HL-60 cells became nonspecific esterase positive cells with 10 nM calcitriol treatment for 3 days [18] as opposed to about 95% of nonspecific esterase positive cells with the same culture condition [25]. These variabilities may be accounted for, in part, by the variability of the serum in which the cells are

grown. Not only does serum contain undefined proteins and factors, but serum can vary widely in its composition from batch to batch.

We have demonstrated that calcitriol caused concentration-dependent maturation of HL-60 cells grown not only in 10% SCM but also in SFM, as evidenced by its ability to decrease cell proliferation, and to induce CL responsiveness and lysozyme production (Figs. 1, 2, 3). Treatment of HL-60 cells with calcitriol resulted in a concentration-dependent increase of α-naphthylacetate esterase enzyme activity (Table 1) which has been shown to occur primarily in monocytes and is virtually absent in granulocytes. In addition, HL-60 cells acquired monocyte-specific cell surface antigens in a dose-dependent manner. These data clearly indicate that HL-60 cells grown in SFM were able to differentiate toward the monocyte/macrophage pathway after exposure to calcitriol. Moreover, the data also suggests that calcitriol directly mediates this differentiation process rather than via a factor(s) contained within serum. These results are consistent with the findings of Breitman et al. [33] who demonstrated that HL-60 cells cultured in serum-free nutrient medium supplemented with transferrin and insulin could differentiate to mature granulocytes in the presence of DMSO. The doses of calcitriol reported in the literature, which have been shown to induce phenotypic changes in HL-60 cells cultured in 10-20% serum supplemented medium, are generally larger than would be predicted by the equilibrium dissociation constant for its receptor [15,18,21,22,43]. This incongruence tends to obfuscate the issue as to whether this hormone is important for "normal" hematopoietic maturation. Furthermore, extrapolating these *in vitro* doses to doses that would be clinically effective for treating hematopoietic disorders, raises serious concern, given the potential toxicities of calcitriol. We sought to address these issues using a

chemically defined medium, not only to assess the dependence of calcitriol-induced differentiation on serum, but also to ascertain the potency of calciol metabolites under conditions that more closely approximated an extracellular environment. In this regard, calcitriol exhibited a 2- to 20-fold increase in potency for inducing several phenotypic changes in HL-60 cells cultured in SFM. Although Table 1 indicates SFM did not potentiate nonspecific esterase response, this is partially due to our use of the appearance or non-appearance of black granules as the assay end point. Microscopic observation of stained slides indicated a quantitative increase in black granulation in calcitriol treated cells cultured in SFM vs. those treated in 10% SCM, but nonuniform cell adhesion, and wide variation in individual cell staining prevented a reliable quantification of this increase.

The increased potency of calcitriol in SFM is consistent with those results obtained by several other laboratories. Ball et al. [43] have demonstrated that HL-60 cells express class 1 HLA antigens when cultured in nutrient medium supplemented with serum and not in a chemically defined serum-free medium. Moreover, calcitriol was able to clearly induce these antigens only in the serum-free medium. Additional corroboration of our results is supported by the findings of Amento et al. [26]. In the absence of serum, changes in U937 cell morphology and decreases in cell proliferation occurred with lower doses of calcitriol than in those cells cultured in the presence of serum. Thus, the phenotypic changes reported by us and others utilizing chemically defined serum-free media occur at concentrations of the active hormone that are more closely associated with physiological concentrations.

Decreased cellular responsiveness in the presence of serum also has been demonstrated for a wide variety of cell types. It has been reported that serum

caused a 5-10 fold decrease in the sensitivity of T-lymphoma cells to growth inhibition by cyclosporin A [44]. In some systems, serum has been shown to inhibit the differentiated function of granulosa cells [45-47]. Moreover, progesterone production in response to FSH was higher in porcine granulosa cells cultured in serum-free medium than in those grown in the presence of 10% fetal calf serum [48].

The lower potency of calcitriol in HL-60 cells cultured in 10% SCM could be a consequence of the vitamin D-binding protein in serum which could decrease the free hormone concentration in the culture medium. This view also has been advanced previously by Amento et al. [26] for calcitriol induced differentiation in U937 cells. In contrast to the uptake of calcitriol in SFM, uptake into intact HL-60 cells is reduced greatly in the presence of 10% fetal calf serum (data not shown). These results are consistent with the hypothesis that serum binding proteins may be acting as a reservoir for the hormone. However, it has been suggested previously that serum binding may not be the only cause for decreases in cell sensitivity to a drug or ligand [44]. Therefore, we cannot exclude the possibility that other substances contained in serum could be antagonizing the differentiating effect of calcitriol in HL-60 cells.

The relative order of potency for calcitriol metabolites causing induction of differentiation in HL-60 cells cultured in SFM was calcitriol >> (1S)-hydroxycalciol > calcidiol ≅ (24R)-hydroxycalcidiol. The inability of calcidiol and (24R)-hydroxycalcidiol to induce differentiation of HL-60 cells in 10% SCM may be due to the fact that these metabolites have less affinity for the calcitriol receptor [15]. In addition, the vitamin D-binding protein in serum binds calcidiol and (24R)-hydroxycalcidiol to a greater degree than calcitriol [49,50], thereby lowering the

concentration of these free calciol metabolites available for entry into the cells. The specificity of these calciol metabolites tested in the present study in inducing differentiation was well correlated with the specificity of their association with the putative calcitriol receptor [15]. The existence of a positive correlation between calcitriolinduced differentiation and the occurrence of occupied calcitriol receptors has been reported previously in HL-60 cells [18]. Likewise, the functional defect of the calcitriol resistant HL-60 clones appears to be correlated with the reduced amount of the specific calcitriol receptor [51]. These results are supportive of the hypothesis that calcitriol induced differentiation in HL-60 cells is receptor mediated [15,18,51], although the mechanism of calcitriol-induced differentiation of HL-60 cells remains unknown.

Since calcitriol was shown to be a more potent cellular differentiating agent when cells were cultured in SFM in comparison to SCM, it was important to examine whether the calcitriol receptor had undergone changes in physical/chemical characteristics. Such structural or chemical differences might be related to the observed differences in hormone potency. For example, other cultured cells grown in serum free media show enhanced numbers of b-adrenergic receptors or changes in regulation of epidermal growth factor receptors [44,52] compared to cells grown in serum containing medium. Culturing HL-60 cells in serum-free conditions does not seem to alter the physical/chemical characteristics of the calcitriol binding protein qualitatively or quantitatively. Similar sedimentation patterns were observed for the [^{3}H]calcitriol receptor in HL-60 cells grown in SFM and SCM. The 3.4S sedimentation coefficient is similar to that reported previously for mammalian calcitriol receptors [1,7]. The calcitriol receptor's inherent DNA-cellulose binding ability also was not affected as indicated by the similar elution patterns of DNA

cellulose chromatography. The equilibrium dissociation constant of calcitriol receptor and the number of binding sites per cell remained unchanged in HL-60 cells cultured in SFM. Thus, it is unlikey that physical/chemical alterations in calcitriol receptors are responsible for the increased potency of calcitriol in HL-60 cells grown in SFM. Moreover, the significant alterations in the physical nature of the receptor might be unlikely from the findings that growth of HL-60 cells in SFM increases the potency but not the efficacy of calcitriol.

In the present study, HL-60 cells that have proliferated in a SFM are still capable of differentiating toward the monocyte/macrophage pathway after exposure to calcitriol. Furthermore, employing SFM for HL-60 cell culture does not alter the physical/chemical characteristics of the calcitriol binding protein qualitatively. Many cell types can now be cultured in completely defined media [32], and the utilization of these systems in the study of molecular mechanism of hormone action may be quite fruitful. Thus further studies can now be pursued utilizing a more "physiological milieu" without the variation introduced by the use of serum.

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Table 1.

Induction of α-naphthylacetate esterase activity in HL-60 cells by calcitriol

Added concentration of calcitriol (nM)	α-Naphthylacetate esterase positive (%)	
	10% SCM	SFM
0.0	<1	<1
0.2	2	3
1.0	6	9
10.0	57	57
50.0	80	63

Cells (4×10^5/ml) were inoculated either in 10% SCM or in SFM and incubated for three days in the presence or absence of various concentrations of calcitriol. Data are expressed as the percentage of total cells assayed and represent the mean of four replications with triplicate measurements.

Table 2.

Monocyte-specific antigen expression on HL-60 cells by calcitriol: indirect immunofluorescence with FACS IV analysis*

Added concentration of calcitriol (nM)		Monoclonal Ab			
		Anti-Leu-M3		Anti-63-D3	
		MFI†	Pbc‡	MFI	Pbc
0.0	10% SCM	46.6	1.1	43.2	0.0
	SFM	50.2	3.5	44.6	1.72
0.5	10% SCM	46.8	2.8	44.5	2.38
	SFM	64.3	36.4	60.2	35.19
5.0	10% SCM	59.1	28.5	58.3	30.88
	SFM	76.8	60.7	72.5	60.97
20.0	10% SCM	75.6	57.0	72.2	59.27
	SFM	86.9	79.7	81.8	81.19

* On the basis of light scatter properties, dead cells were excluded from fluorescence analysis. Light scatter windows were also chosen to maximize purity of the analyzed populations.

† MFI. Normalized mean fluorescence intensity

‡ Pbc. Percentage of cells brigher than negative control fluo-rescence.

Figure 1. Effect of calcitriol on cell growth of HL-60 cells cultured in 10% SCM (open symbols) (A) and in SFM (filled symbols) (B): Cells were inoculated at 2×10^5 cells/ml in duplicate wells and incubated for four days with 0.1% ethanol (O, ●) (control), or calcitriol 0.2 nM (❑, ■), 5.0 nM (Δ, ▲) or 10.0 nM (∇, ▼). On the days indicated, cells were harvested and the viable cell number was counted as described in Materials and Methods section. Each point is a mean ± SE of duplicate measurements from average of eight separate experiments.

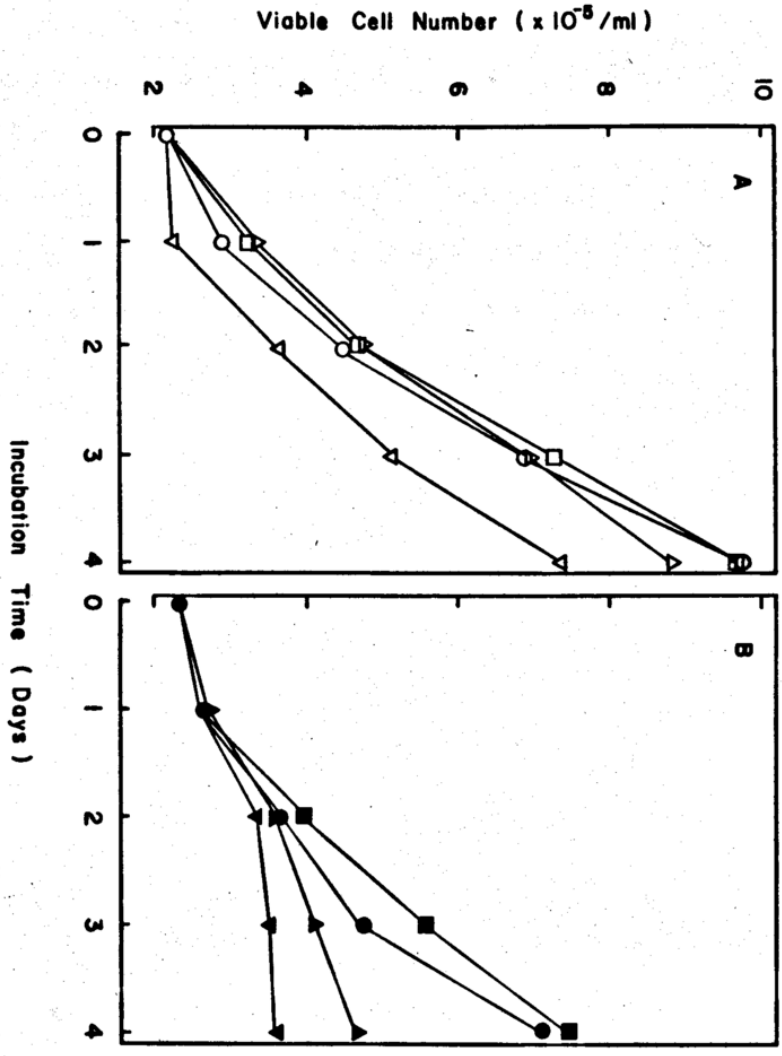

Viable Cell Number (x 10⁻⁵ /ml)
2
4
6
8
10
A
B
0
1
2
3
4
Incubation Time (Days)

Figure 2. Effect of calciol metabolites on induction of CL responses in HL-60 cells cultured in either 10% SCM or in SFM: Cells (4×10^5/ml) were incubated with various concentrations of calcitriol (O, ●), (1S)-hydroxycalciol (Δ, ▲), (24R)-hydroxycalcidiol (∇, ▼), or calcidiol (❑, ■) for three days. Open symbols and closed symbols represent CL responses in cells cultured in 10% SCM and SFM, respectively. CL levels for 10^6 cells were determined 10 min after PMA was added to the reaction mixture as described in Materials and Methods section. Values represent the means ± SE of duplicate determinations from an average of five replications.

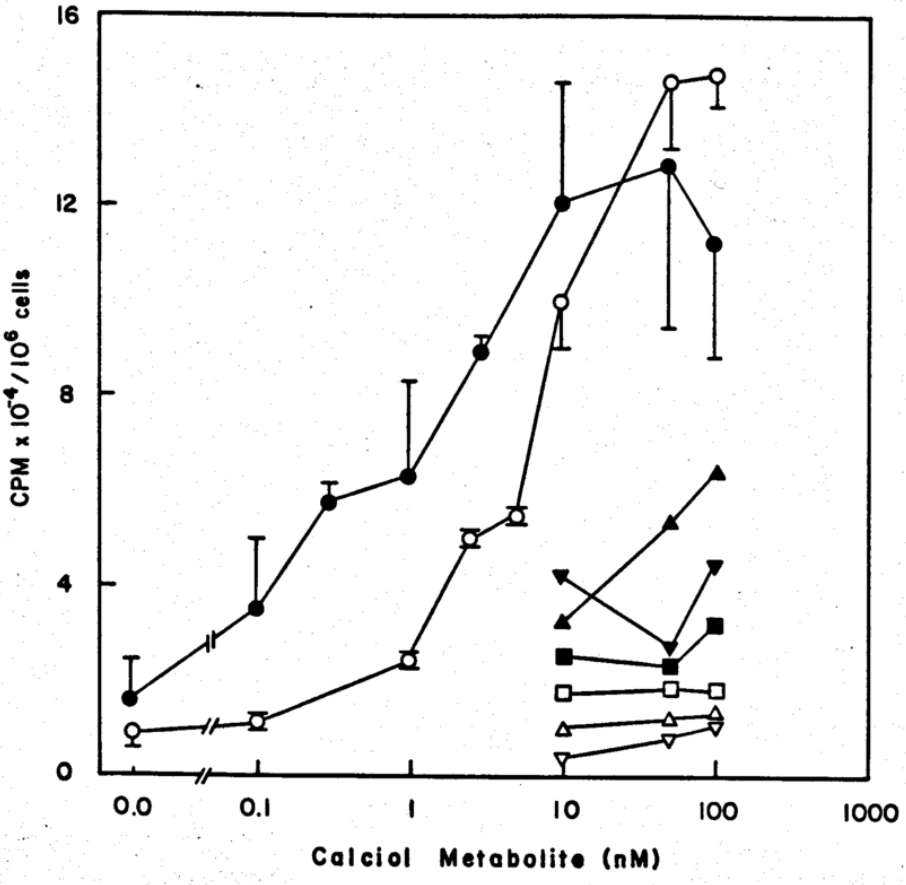

16
12
8
4
0
CPM x 10⁻⁴/10⁶ cells
0.0
0.1
1
10
100
1000
Calciol Metabolite (nM)

Figure 3. Induction of extracellular lysozyme by calciol metabolites in HL-60 cells cultured either in 10% SCM or in SFM: The cells (4×10^5/ml) were incubated with various concentrations of calcitriol (O, ●), (1S)-hydroxycalciol (Δ, ▲), (24R)-hydroxycalcidiol (∇, ▼), or calcidiol (❑, ■) for three days; open symbols for 10% SCM and closed symbols for SFM. The data represent the means ± SE of triplicate measurements from the average of six replications.

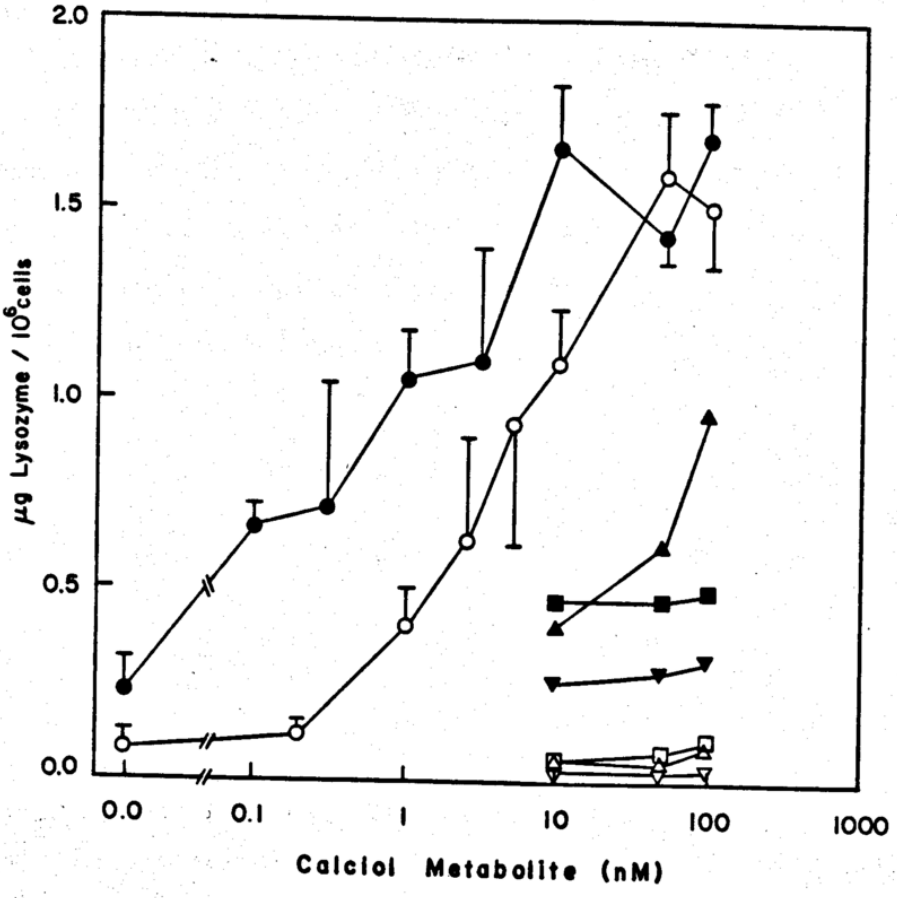
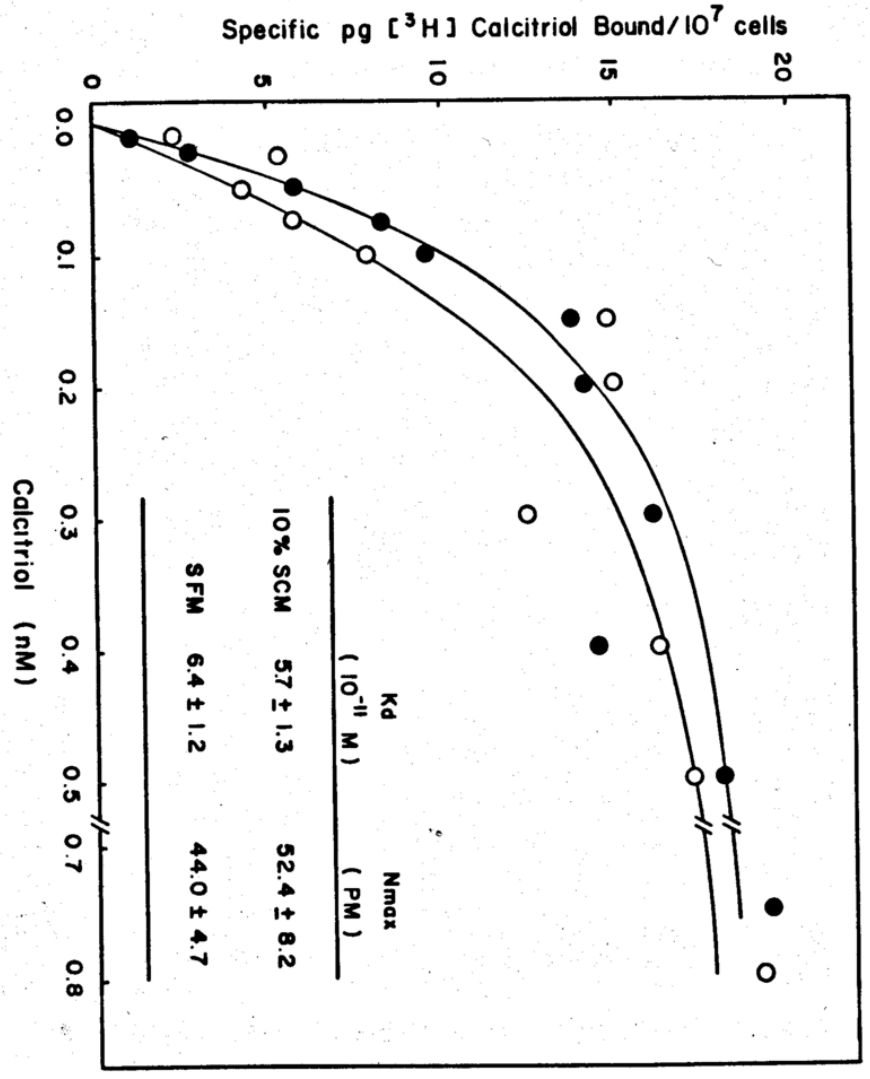

2.0
1.5
1.0
0.5
0.0
μg Lysozyme / 10^6 cells
0.0
0.1
1
10
100
1000
Calciol Metabolite (nM)

Figure 4. Determination of the apparent equilibrium dissociation constant (K_d) for [^{3}H]calcitriol binding to receptors in intact HL-60 cells cultured in 10% SCM (O) and in SFM (●). Receptor-bound [^{3}H]calcitriol was quantitated by an HAP batch assay as described in Materials and Methods section. Values represent the means of four to six replications with duplicate determinations. The apparent K_d for specific [^{3}H]calcitriol binding calculated from the slopes of the regression lines were 5.7×10^{-11} and 6.4×10^{-11} for HL-60 cells in 10% SCM and SFM, respectively, while the concentrations of specific binding sites estimated from the abscissa intercepts were ~3,000 binding sites for a HL-60 cell cultured in either in 10% SCM or in SFM (see inset for Scatchard transformation).


Specific pg [3H] Calcitriol Bound/10^7 cells
0
5
10
15
20
0.0
0.1
0.2
0.3
0.4
0.5
0.7
0.8
Calcitriol (nM)
Kd (10^-11 M)
Nmax (PM)
10% SCM 5.7 ± 1.3 52.4 ± 8.2
SFM 6.4 ± 1.2 44.0 ± 4.7