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1α,25-Dihydroxyvitamin D_3 Modulation in Lipid Metabolism in Established Bone Marrow-Derived Stromal Cells, MC3T3-G2/PA6

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Abstract MC3T3-G2/PA6 (PA6) cells established from newborn mouse calvaria are preadipocytic stromal cells, which differentiate into adipocytes in response to glucocorticoids. We examined the effects of 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] on adipogenesis in PA6 cells. When PA6 cells were cultured with 10^{-8} M dexamethasone, adipocytes containing oil red O-positive droplets first appeared on day 7 (3 days after confluence was attained) and the maximal synthesis of neutral lipids occurred on day 12. Simultaneous addition of 1α,25$(OH)_2D_3$ at 10^{-9} M completely blocked this dexamethasone-induced neutral lipid synthesis throughout the 14-day culture period. Dose-response studies of vitamin D_3 derivatives showed that 1α,25$(OH)_2D_3$ was the most potent in inhibiting neutral lipid synthesis in PA6 cells, followed by 1α-hydroxyvitamin D_3, 25-hydroxyvitamin D_3, and 24R,25-dihydroxyvitamin D_3, in that order. Dexamethasone greatly enhanced incorporation of [^{14}C]-acetic acid into triacylglycerol in PA6 cells. The incorporation was markedly inhibited by the addition of 10^{-9} M 1α,25$(OH)_2D_3$. Instead, 1α,25$(OH)_2D_3$ greatly increased incorporation of [^{14}C]-acetic acid into phospholipids, such as phosphatidylcholine and phosphatidylethanolamine, irrespective of the presence or absence of dexamethasone. These results suggest that 1α,25$(OH)_2D_3$ modulation of lipid metabolism in bone marrow stromal cells is receptor mediated.

Key words: adipocyte, adipogenesis, osteoporosis, phospholipids, preadipocyte, triacylglycerol, vitamin D derivatives

It is well established that vitamin D_3 is metabolized first in the liver to 25-hydroxyvitamin D_3 [25$(OH)D_3$] (Ponchon et al., 1969), then in the kidney, mainly to 24R,25-dihydroxyvitamin D_3 [24R,25$(OH)_2D_3$] (Holick et al., 1972) and 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] (Holick et al., 1971; Lawson et al., 1971; Norman et al., 1971). Of these metabolites, 1α,25$(OH)_2D_3$ is considered to be the hormonal form of the vitamin which functions in regulating blood calcium levels by enhancing intestinal calcium transport and bone mineral mobilization activities (DeLuca, 1988; Suda et al., 1990). Furthermore, recent reports have suggested that 1α,25$(OH)_2D_3$ is a regulator of not only mineral metabolism but also growth and differentiation of hemopoietic cells (Miyaura et al., 1982; Dorshkind et al., 1989), malignant cells (Abe et al., 1981; Tanaka et al., 1982), skin epidermal cells (Hosomi et al., 1983; Smith et al., 1986), and preadipocytes (Sato and Hiragun, 1988).

MC3T3-G2/PA6 (PA6) cells established from the calvaria of newborn mice are preadipocytic stromal cells (Kodama et al., 1982a). Glucocorticoids but not insulin stimulate differentiation of PA6 cells into adipocytes (Kodama et al., 1982a). It has been reported that PA6 cells are capable of supporting in vitro proliferation of hemopoietic stem cells, defined as CFU-S (colony-forming units in spleen), in co-cultures with bone marrow cells (Kodama et al., 1982b, 1984, 1986). We previously reported that PA6 cells supported formation of osteoclast-like multinucleated cells in co-cultures with mouse spleen cells in the presence of 1α,25$(OH)_2D_3$ (Udagawa et al., 1989). Simultaneous addition of dexamethasone to the co-cultures markedly enhanced the osteoclast-like cell formation induced by 1α,25$(OH)_2D_3$ (Udagawa et al., 1989). In the course of examining the effect of 1α,25$(OH)_2D_3$ on the differentiation of PA6 cells, we noticed that 1α,25$(OH)_2D_3$ inhib-

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ited the induction of oil red O-positive droplets by dexamethasone in PA6 cells.

In the present study, we investigated the effects of $1\alpha,25(OH)_2D_3$ on the differentiation of PA6 cells into adipocytes. Its effects on lipid metabolism in these cells were also examined in greater detail. Treatment of PA6 cells with dexamethasone enhanced triacylglycerol synthesis, which was strongly inhibited by $1\alpha,25(OH)_2D_3$. Interestingly, $1\alpha,25(OH)_2D_3$ stimulated synthesis of phospholipids irrespective of the presence or absence of dexamethasone. These findings suggest that $1\alpha,25(OH)_2D_3$ plays an important role in lipid metabolism in bone marrow-derived stromal cells.

MATERIALS AND METHODS

Chemicals

Dexamethasone was purchased from Sigma Chemical Co. (St. Louis, MO). $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ were purchased from Phillips-Dupher (Amsterdam, The Netherlands). $24R,25(OH)_2D_3$ was kindly provided by Kureha Chemical Industry (Tokyo, Japan). 1α-Hydroxyvitamin D_3 [$1\alpha(OH)D_3$] was the gift of Dr. I. Matsunaga (Chugai Pharmaceutical Co., Tokyo). Standard lipids, such as triacylglycerol, diacylglycerol, cholesterol, free fatty acids, and a phospholipid kit, were obtained from Sergary Research Laboratories (Ontario, Canada). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY). [^{14}C]-Acetic acid (sodium salt; 7.4×10^7 Bq/mmol) was obtained from New England Nuclear (Boston, MA). Alpha-minimal essential medium (α-MEM) was purchased from Flow Laboratories (McLean, VA). Culture dishes and plates were obtained from Corning (Corning, NY). Thin-layer plates (precoated silica gel 60 plates) were purchased from Merck (Darmstadt, West Germany).

Cell Cultures

A stock of the clonal preadipocytic stromal cell line, MC3T3-G2/PA6 (PA6), established from the calvaria of newborn C57BL/6 mice was kindly supplied by Dr. Kodama (Ohu University, Fukushima, Japan). PA6 cells were maintained in α-MEM containing 10% FBS. The conversion of PA6 cells to adipocytes was induced by the addition of 10^{-8} M dexamethasone as this concentration most effectively induced the formation of oil red O-positive droplets in PA6 cells after the cells had been cultured for 12 days. To determine the effects of vitamin D_3 derivatives on the conversion of PA6 cells to adipocytes, the PA6 cells were cultured in 24-well plates at 1×10^4 cells/well (0.5 ml/well). Cultures were fed every 3 days by replacing 0.4 ml of old medium with the same volume of fresh medium. Various concentrations of $1\alpha,25(OH)_2D_3$, $1\alpha(OH)D_3$, $25(OH)D_3$, and $24R,25(OH)_2D_3$ were added at the beginning of the culture and at the time of each medium change. At the ends of the indicated culture periods (usually 12 days), cells were fixed with 10% formalin in phosphate buffered saline (PBS), stained with oil red O for 1 h, as described by Kuri-Harcuch and Green (1978), and counterstained with hematoxylin. Adipocyte and total cell numbers were determined in five randomly selected 0.46 mm^2 areas of the well. Data are expressed as the means ± SEM of three or four cultures.

Lipid Metabolism

PA6 cells were cultured in 100 mm plastic dishes (5×10^5 cells/dish) in the presence or absence of dexamethasone, $1\alpha,25(OH)_2D_3$, or both. After 12 days, the culture medium was replaced with 8 ml of fresh medium containing [^{14}C]-acetic acid (1.85×10^5 Bq/ml). Following incubation for 3 h at 37°C, the cell layer was washed three times with PBS and homogenized in 5 ml of PBS with a sonicator. Lipids were then extracted with chloroform-methanol (2:1, v/v) according to the method of Bligh and Dyer (1959). The chloroform layer was collected and evaporated under nitrogen gas. The lipid extracts were dissolved in chloroform-methanol (2:1, v/v). The samples and various standards were applied quantitatively with a microsyringe to silica gel plates (10 × 20 cm, 0.2 mm in thickness). The plates were first developed with a first solvent system composed of chloroform: methanol:acetic acid:formic acid:water (35:15:6:1:2, v/v), followed by a second solvent system consisting of n-hexane:diethyl ether:water (39:10:1, v/v). For two-dimensional thin-layer chromatography, the total extracted lipid contents of the PA6 cells were applied to silica gel plates (10 × 10 cm, 0.2 mm in thickness). The plates were developed with chloroform:methanol:28% aqueous ammonia (13:7:1, v/v) for development of the first dimension and with chloroform: acetone:methanol:acetic acid:water (10:4:2:2:1, v/v) for the second. After development, the standard's spots were visualized by exposing the

plates to iodine vapor. The plates of samples were blown with a surface autoradiography enhancer for one-step rapid visualization of low-level beta-emitting materials isolated on the plates, then exposed to Kodak X-Omat AR x-ray films (Eastman Kodak, Rochester, NY) at −80°C. Finally, each spot on the plates was scraped off and its radioactivity was measured.

RESULTS

When PA6 cells were cultured with 10^{-8} M dexamethasone, many oil red O-positive droplets appeared in the cells on day 12 (Fig. 1B). Simultaneous treatment of the cultures with 10^{-9} M $1\alpha,25(OH)_2D_3$ completely inhibited the dexamethasone-induced appearance of oil red O-positive droplets (Fig. 1C). $1\alpha,25(OH)_2D_3$ per se induced no appreciable morphological changes in the cells (Fig. 1D).

Figure 2 shows the time courses of changes in growth and adipocyte conversion in PA6 cells cultured with dexamethasone and $1\alpha,25(OH)_2D_3$, separately or in combination. PA6 cells seeded at 1×10^4 cells/well grew rapidly and reached confluence on day 4 at a density of 8×10^4 cells/well in control cultures. The growth rate and cell density at confluence were not noticeably affected by the addition of 10^{-8} M dexamethasone, 10^{-9} M $1\alpha,25(OH)_2D_3$, or 10^{-8} M dexamethasone plus 10^{-9} M $1\alpha,25(OH)_2D_3$. In cultures treated with dexamethasone, adipocytes first appeared on day 7 and maximal adipocyte conversion occurred on day 12. A small number of adipocytes were detected in the control cultures on day 12. When 10^{-9} M $1\alpha,25(OH)_2D_3$ was added to the PA6 cultures, appearance of oil red O-positive droplets was completely inhibited throughout the 14 day culture period, even in the presence of 10^{-8} M dexamethasone. In 17 independent experiments, the numbers of adipocytes/well formed on day 12 were as follows: control, 326 ± 70/well (range 0–835/well); dexamethasone 10^{-8} M, 5680 ± 909/well (range, 1,300–9,228/well); 10^{-9} M dexamethasone plus 10^{-9} M $1\alpha,25(OH)_2D_3$, 0; 10^{-8} M $1\alpha,25(OH)_2D_3$, 0.

Figure 3 shows dose-response effects of vitamin D_3 derivatives on adipocyte conversion of PA6 cells treated with 10^{-8} M dexamethasone. All vitamin D_3 derivatives examined dose-dependently inhibited the dexamethasone-induced conversion of PA6 cells to adipocytes. $1\alpha,25(OH)_2D_3$ was the most potent inhibitor, followed by $1\alpha(OH)D_3$, $25(OH)D_3$, and $24R,25(OH)_2D_3$, in that order. The dexamethasone-induced adipocyte conversion was significantly inhibited by 10^{-10} M $1\alpha,25(OH)_2D_3$ and completely blocked by 10^{-9} M. These results suggest that vitamin D-induced inhibition of the conversion of PA6 cells to adipocytes is mediated by $1\alpha,25(OH)_2D_3$ receptors.

Figure 4 shows the autoradiography of lipid components produced by PA6 cells which had been incubated with [^{14}C]-acetic acid, and Table I summarizes the results of the radioactivities

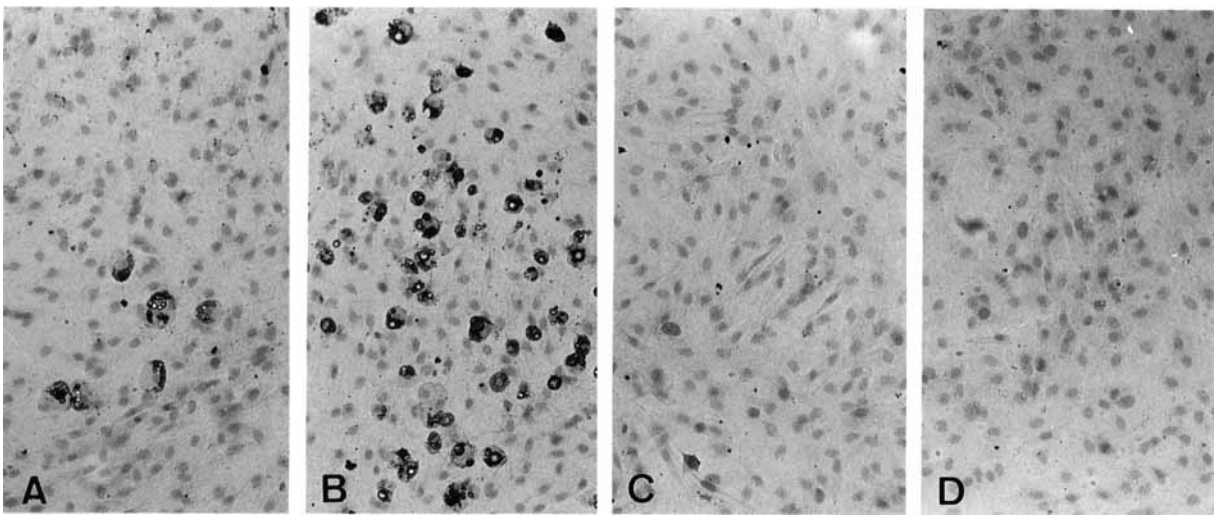

Fig. 1. Histochemistry of adipocyte conversion of PA6 cells. PA6 cells were cultured with (A) vehicle, (B) 10^{-8} M dexamethasone, (C) 10^{-8} M dexamethasone plus 10^{-9} M $1\alpha,25(OH)_2D_3$, or (D) 10^{-9} M $1\alpha,25(OH)_2D_3$. After being cultured for 12 days, cells were fixed, stained with oil red O, and counterstained with hematoxylin. Cells which had converted to adipocytes stained red with oil red O. Note that dexamethasone stimulates adipocyte conversion of PA6 cells and that $1\alpha,25(OH)_2D_3$ completely inhibits this dexamethasone-induced conversion. Magnification, × 100.

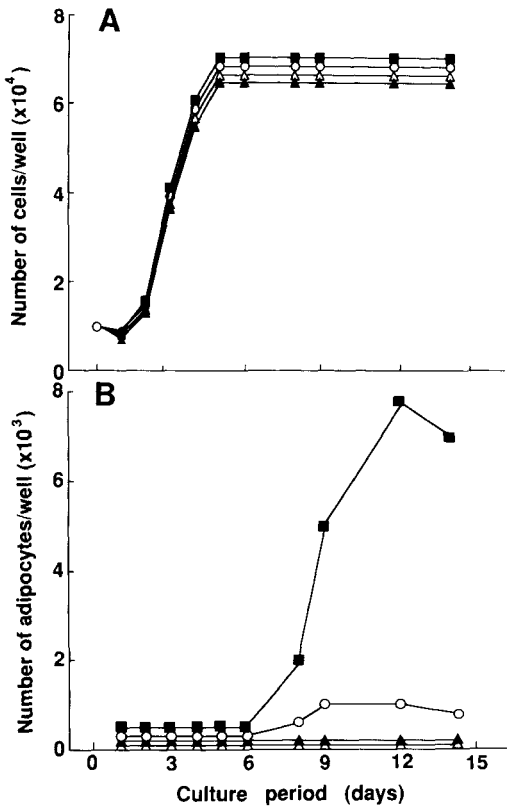

Fig. 2. Time course of changes in the growth (A) and adipocyte conversion (B) of PA6 cells. Cells were plated onto 24-well plates at 1 × 10^4 cells/well and cultured with vehicle (○), 10^{-8} M dexamethasone (■), 10^{-9} M 1α,25(OH)$_2$D$_3$ (△), or 10^{-8} M dexamethasone plus 10^{-9} M 1α,25(OH)$_2$D$_3$ (▲). At the indicated times, cells were fixed, stained with oil red O, and counterstained with hematoxylin. Total cell nuclei and adipocytes were counted in five randomly selected fields (0.46 mm^2) of the well. Results are expressed as the means of three cultures. Similar levels of time-dependent growth and adipocyte conversion were observed in two additional independent experiments.

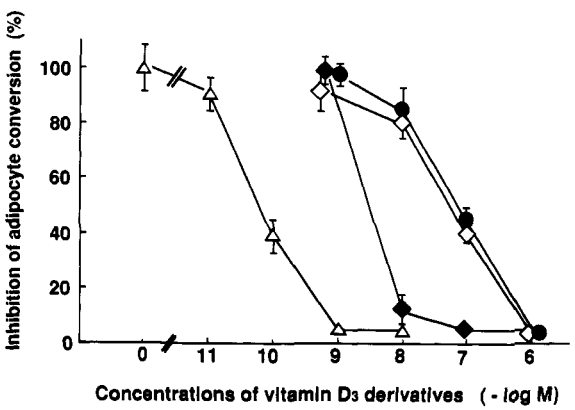

Fig. 3. Effects of increasing concentrations of vitamin D$_3$ derivatives on dexamethasone-induced conversion of PA6 cells to adipocytes. PA6 cells (1 × 10^4 cells/well) were cultured in the presence of 10^{-8} M dexamethasone. Increasing concentrations of 1α,25(OH)$_2$D$_3$ (△), 1α(OH)D$_3$ (◆), 25(OH)D$_3$ (◇), or 24R,25(OH)$_2$D$_3$ (●) were added to the cultures. After being cultured for 12 days, cells were fixed and stained with oil red O, and numbers of adipocytes were scored. Results are expressed as the percentages of adipocytes formed in experimental cultures to those in the cultures treated with 10^{-8} M dexamethasone. The number of adipocytes formed in the dexamethasone-treated cultures was 5716 ± 168/well (the mean ± SEM of four cultures).

incorporated into the triacylglycerol and phospholipid spots shown in Figure 4. PA6 cells cultured with dexamethasone for 12 days exhibited markedly increased triacylglycerol synthesis (Fig. 4A lane 2, and Fig. 4B lane 2; Table I). The dexamethasone-induced triacylglycerol synthesis was markedly inhibited by simultaneous addition of 10^{-9} or 10^{-8} M 1α,25(OH)$_2$D$_3$ (Fig. 3, Table I). In contrast, 1α,25(OH)$_2$D$_3$ stimulated the synthesis of phospholipids irrespective of the presence or absence of dexamethasone (Fig. 4, and Table I).

To characterize the phospholipids induced by 1α,25(OH)$_2$D$_3$, we performed two-dimensional thin-layer chromatography of lipid extracts obtained from PA6 cells. As shown in Figure 5, syntheses of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) were stimulated to varying degrees by the addition of 10^{-8} M 1α,25(OH)$_2$D$_3$ (Fig. 4). Stimulation of PC synthesis, which occurred in response to the addition of 10^{-9} M 1α,25(OH)$_2$D$_3$, was the most sensitive (Fig. 5). Dexamethasone at 10^{-8} M slightly stimulated phospholipid synthesis in control cultures, but had little effect on the phospholipid synthesis induced by 1α,25(OH)$_2$D$_3$ (Fig. 5).

DISCUSSION

The results of the present study clearly demonstrate that 1α,25(OH)$_2$D$_3$ inhibits the dexamethasone-induced conversion of PA6 cells to adipocytes (Figs. 1, 2). Other vitamin D$_3$ derivatives, such as 1α(OH)D$_3$, 25(OH)D$_3$, and 24R,25(OH)$_2$D$_3$, similarly inhibited adipocyte conversion of PA6 cells, but much higher concentrations were required (Fig. 3). The potencies of vitamin D$_3$ derivatives in inhibiting adipocyte conversion correlated to their binding affinities to 1α,25(OH)$_2$D$_3$ receptors (DeLuca, 1988). Therefore, it appears that the 1α,25(OH)$_2$D$_3$-induced inhibition of the conversion of PA6 cells to adipocytes is controlled by a classical receptor-mediated mechanism. This notion is

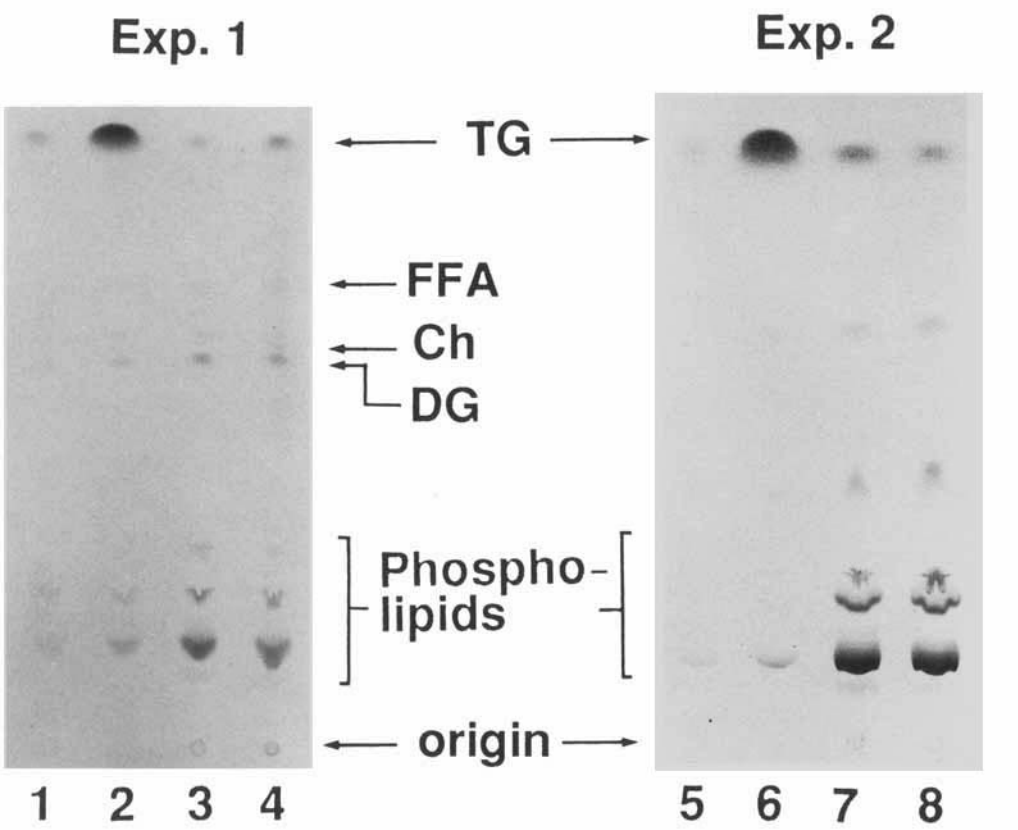

Fig. 4. Autoradiography of lipid components produced by PA6 cells which had been incubated with [^{14}C]-acetic acid. **Exp. 1:** PA6 cells were plated onto 100 mm dishes at 5×10^5 cells/dish and cultured with vehicle (lane 1), 10^{-8} M dexamethasone (lane 2), 10^{-8} M dexamethasone plus 10^{-9} M 1α,25(OH)$_2$D$_3$ (lane 3), or 10^{-9} M 1α,25(OH)$_2$D$_3$ (lane 4). **Exp. 2:** PA6 cells were similarly cultured with vehicle (lane 5), 10^{-8} M dexamethasone (lane 6), 10^{-8} M dexamethasone plus 10^{-8} M 1α,25(OH)$_2$D$_3$ (lane 7) or 10^{-8} M 1α,25(OH)$_2$D$_3$ (lane 8). After being cultured for 12 days, the cells were incubated for 3 h with [^{14}C]-acetic acid (1.85×10^5 Bq/ml). Lipids were then extracted, analyzed by thin-layer chromatography, and processed for autoradiography. The arrows indicate the positions of the standards: triacylglycerol (TG), free fatty acids (FFA), cholesterol (Ch), diacylglycerol (DG), and phospholipids.

supported by our preliminary observation that PA6 cells possess specific binding molecules for 1α,25(OH)$_2$D$_3$ (Kd, 1.66×10^{-10} M; maximal binding, 120 fmol/mg protein).

The 1α,25(OH)$_2$D$_3$-induced inhibition of the conversion of PA6 cells to adipocytes was accompanied by a marked increase in acetic acid incorporation into the phospholipid fraction, especially into phosphatidylcholine and phosphatidylethanolamine (Table I, Fig. 5). The 1α,25(OH)$_2$D$_3$-stimulated increase in phospholipid synthesis occurred irrespective of the presence or absence of dexamethasone. This suggests that the effect of 1α,25(OH)$_2$D$_3$ on phospholipid metabolism is independent of the 1α,25(OH)$_2$D$_3$-induced inhibition of the conversion of preadipocytes to mature adipocytes. These results are in agreement with previous observations in other cell systems. Rasmussen et al. (1982) reported that the administration of 1α,25(OH)$_2$D$_3$ to vitamin D-deficient chicks caused an increase in the synthesis of phosphatidylcholine in brush-border membranes prepared from duodenal mucosal cells. Treatment with 1α,25(OH)$_2$D$_3$ has also been observed to induce an increase in phosphatidylcholine production in promyelocytic leukemia cells (HL-60) (Levy et al. 1987). Tsutsumi et al. (1984) reported that the phospholipid content of renal brush-border membranes in vitamin D-deficient rats was significantly lower than that in vitamin D-supplemented animals and that the administration of 1α,25(OH)$_2$D$_3$ to vitamin D-deficient rats normalized the phospholipid content within 16 h. Matsumoto et al. (1985) have also shown that 48 h treatment of osteoblastic osteosarcoma cells (ROS 17/2.8) with 1α,25(OH)$_2$D$_3$ caused significant changes in phospholipid metabolism. 1α,25(OH)$_2$D$_3$ enhanced incorporation of [^{14}C]-serine into phosphatidylserine, but sup-

TABLE I. Effects of Dexamethasone and 1α,25(OH)$_2$D$_3$ on Triacylglycerol and Phospholipid Synthesis in PA6 Cells*

Treatment	Lipid fractions: Triacylglycerol	Lipid fractions: Phospholipids
Experiment 1	(cpm/dish)	
Vehicle	281	128
Dexamethasone (10^{-8} M)	8827	323
Dexamethasone (10^{-8} M) + 1α,25(OH)$_2$D$_3$ (10^{-9} M)	224	1308
1α,25(OH)$_2$D$_3$ (10^{-9} M)	976	2155
Experiment 2		
Vehicle	553	383
Dexamethasone (10^{-8} M)	5792	588
Dexamethasone (10^{-8} M) + 1α,25(OH)$_2$D$_3$ (10^{-8} M)	1704	4939
1α,25(OH)$_2$D$_3$ (10^{-8} M)	1187	5364

*Each triacylglycerol and phospholipid fraction spot on the thin-layers shown in Figure 4 was scraped off and radioactivity counts were determined. The results are expressed as cpm/dish.

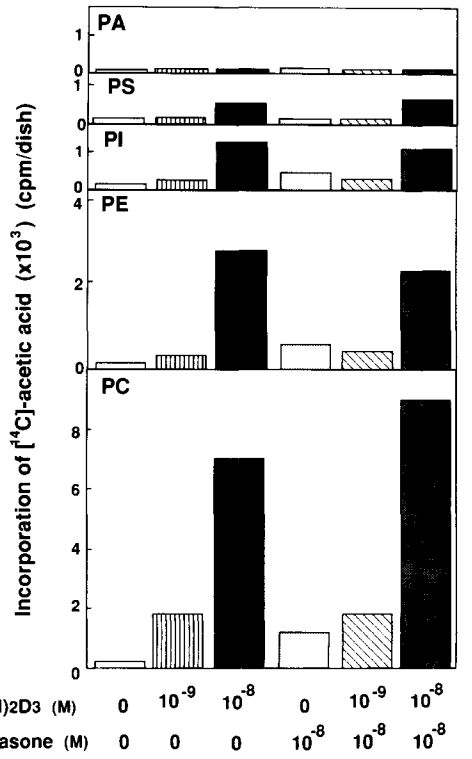

Fig. 5. Effects of dexamethasone and 1α,25(OH)$_2$D$_3$ on phospholipid synthesis in PA6 cells. PA6 cells were plated onto 100 mm dishes at 5 × 10^5 cells/dish and cultured with or without 10^{-8} M dexamethasone in the presence or absence of either 10^{-9} M or 10^{-8} M 1α,25(OH)$_2$D$_3$. After being cultured for 12 days, the cells were incubated for 3 h with [^{14}C]-acetic acid (1.85 × 10^5 Bq/ml). Lipids were then extracted and separated by two-dimensional thin-layer chromatography. The radioactivities incorporated in the respective spots of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidic acid (PA) were counted. The radioactivity incorporated into the PA fraction was below the detection limit (less than 30 cpm/dish) in each dish.

pressed incorporation of [^{3}H]-ethanolamine into phosphatidylethanolamine (Matsumoto et al., 1985). Thus, 1α,25(OH)$_2$D$_3$ regulates phospholipid metabolism in a variety of cells and tissues, suggesting that such changes in phospholipid components are somehow involved in mediating the biological functions of 1α,25(OH)$_2$D$_3$.

There are two types of adipocyte conversion: One is induced by insulin and the other by glucocorticoids. Sato and Hiragun (1988) reported that adipocyte conversion of ST13 and 3T3-L1 preadipocytes is induced by insulin and it is strongly inhibited by 1α,25(OH)$_2$D$_3$. This inhibition also appears to be modulated by a 1α,25(OH)$_2$D$_3$ receptor-mediated mechanism. Thus, 1α,25(OH)$_2$D$_3$ inhibits conversion of both types of preadipocytes. These observations suggest that 1α,25(OH)$_2$D$_3$ acts as an inhibitor of adipocyte conversion in a wide range of tissues in vivo.

Kodama et al. (1984) reported that adipocytes which had differentiated from PA6 cells in response to dexamethasone could not support the growth of hematopoietic stem cells in co-cultures with bone marrow cells. This finding is consistent with the fact that hemopoiesis occurs actively in red marrow but not in yellow marrow, which consists mainly of adipocytes (Weiss et al., 1988). We also found that the addition of dexamethasone to mouse primary marrow cultures stimulated the conversion of stromal cells to adipocytes and that this conversion was completely inhibited by the addition of 10^{-9} M 1α,25(OH)$_2$D$_3$ (unpublished data). It is, therefore, likely that 1α,25(OH)$_2$D$_3$ is involved in bone marrow hemopoiesis. This involvement is reflected, at least in part, by 1α,25(OH)$_2$D$_3$ regulation of adipocyte conversion of marrow-derived stromal cells.

It is known that the fat content of bone marrow increases with age, particularly in parallel with worsening osteoporosis. Meunier et al. (1971) compared osteoporotic patients and age-matched healthy subjects in terms of marrow fat content in iliac crest biopsy specimens. The fat content was much higher in the osteoporotic

patients than in age-matched healthy subjects. Recently, Martin et al. (1991) demonstrated a negative correlation between fat contents and bone formation ratios in metaphyseal specimens from osteoporotic women. Therefore, administration of $1\alpha,25(OH)_2D_3$ to osteoporotic patients may be useful for preventing the progression of osteoporosis.

In summary, $1\alpha,25(OH)_2D_3$ markedly inhibits dexamethasone-induced adipocyte conversion of marrow-derived preadipocytes, PA6 cells. $1\alpha,25(OH)_2D_3$ does, however, have a strong stimulatory effect on phospholipid synthesis in PA6 cells, irrespective of the presence or absence of dexamethasone. Further studies are required to elucidate the mechanism of action of $1\alpha,25(OH)_2D_3$ in adipocyte conversion and the physiological significance of altered lipid metabolism due to $1\alpha,25(OH)_2D_3$ action on vitamin D target cells.

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