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Effects of a New 1,25(OH)2-Vitamin D3 Analog on Proliferation and Differentiation of the Human Histiocytic Lymphoma Cell Line U937

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Abstract

This study describes the effects of a novel 1,25-dihydroxyvitamin D3 analog [1,25(OH)2-16ene-23yne-26,27-F6-D3] on proliferation and differentiation of the human histiocytic lymphoma cell line U937 *in vitro*. We also examined the expression of c-*myc* oncogene in U937 cells. Growth of U937 cells was apparently inhibited to 62% and 87% of the control level after 4 days in the presence of 10^{-8}M and 10^{-7}M of this analog, respectively. This compound morphologically and functionally differentiated U937 cells to monocyte-macrophage phenotype showing the increase of adherence ability to surface and a decrease of N/C ratio in Giemsa staining. Especially, nonspecific esterase activity which is a marker of cell differentiation to monocyte-macrophage was positive, and production of the positive stained cells increased in a dose dependent fashion. The expression of c-*myc* oncogene by 1,25(OH)2D3 analog (10^{-7}M) was reduced by 60% at the mRNA level as determined by Northern blotting. The effects of this novel analog on cell proliferation and cell differentiation may open up new therapeutic strategies for human disorders such as psoriasis and may provide a tool to understand the mechanism of action of vitamin D3 seco-steroids in malignancy.

Key words : vitamin D3 analog [1,25 (OH)2-16ene-23yne-26,27-F6-D3], U937 leukemia cell differentiation, c-*myc* oncogene expression

INTRODUCTION

The hormonally active form of vitamin D3, 1α, 25-dihydroxyvitamin D3[1,25(OH)2D3], results from sequential hydroxylation in the liver and kidney of parent vitamin D3 (Fig. 1). It plays an important role in the maintenance of calcium homeostasis through binding to specific intracellular receptors[1,2]. The classic target organs of this seco-steroid are the intestine, bone and kidney. A variety of tissues that do not participate in mineral and skeletal metabolism also posses specific receptor for and respond to the hormonal signal of 1, 25(OH)2D3[3~5]. These new discoveries have made apparent that this hormone plays a much larger role in biology including cellular proliferation and differentiation of hematopoietic cells[6,7]. The 1,25(OH)2D3 induces differentiation of acute myelogenous leukemic (AML) lines into monocyte macrophage like cells *in vitro*[6~10]. In addition, clonogenic blast cells from many patients with acute myelogenous leukemia are inhibited in their proliferation by 1,25(OH)2D3 and many are induced to differentiate into macrophage-like cells[10,11]. In contrast, normal human granulocyte-monocyte committed stem cells (GM-CSC) are slightly stimulated in their clonal proliferation by 1,25 (OH)2D3[10,11]. Studies *in vivo* suggest that 1,25 (OH)2D3 can prolong the survival of mice inoculated with syngeneic leukemic cells[12,13]. However, to date, little is known about the possible therapeutic utilization of 1, 25 (OH)2D3 and its analogs in human malignancies. 1,25 (OH)2D3 was administered to preleukemic patients to improve their hematopoiesis. No significant improvement was seen, because hypercalcemia prevented the administration of high dose of 1,25 (OH)2D3[14]. Many investigators have therefore turned their attention to the development of new vitamin D3 analogs with greater specificity in their antimalignancy effects and lesser hypercalcemia properties[15~17].

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Some of these new analogs are 25-OH-16ene-23yne-26,27-F_6-D_3, 1α-F-25-OH-16ene-23yne-26,27-F_6-D_3, 1α-(OH)-25-Oxo-25-phospha-D_3 and 24-Nor-1,25-$(OH)_2$-D_3 containing 1,25-$(OH)_2$-16ene-23yne-26,27-F_6-D_3(Fig. 2). Among these new analogs, 1,25-$(OH)_2$-16ene-23yne-26,27-F_6-D_3 was found to have prominent effects on growth inhibition and differentiation of HL-60 cells with less ability to induce hypercalcemia as compared to the reference, 1,25 $(OH)_2D_3$[38].

In the present paper, we describe the effects of a novel 1,25-dihydroxyvitamin D_3 analog [1,25 $(OH)_2$-16ene-23yne-26,27-F_6-D_3] on induction of cell differentiation and inhibition of cell proliferation using the human histiocytic lymphoma cell line U937. The effect of this compound on expression of c-*myc* oncogene in U937 cell line was also investigated.

MATERIALS AND METHODS

Cells

U937 cells were provided by American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 medium (Gibco Co.) supplemented with 10% heat-inactivated fetal bovine serum (Gibco Co.) at 37°C in a 5% CO_2 humidified atmosphere. Bone marrow was obtained from healthy human volunteers by aspiration after informed written consent was obtained. Their mononuclear cells were isolated by centrifugation on Ficoll-Hypaque gradients, washed twice in 1 × phosphate buffered saline (PBS) and suspended in RPMI 1640 containing 10% fetal bovine serum.

Vitamin D_3 analog preparation

1,25$(OH)_2$-16ene-23yne-26,27-F_6-D_3 was obtained as a gift from Dr. Milan Uskokovic (Hoffmann-La Roche Inc., Nutley, NJ). The chemical structure of the 1,25-$(OH)_2$-16ene-23yne-26,27-F_6-D_3 is shown in Fig. 2. The compound was dissolved in absolute ethanal at 10^{-3}mol/L based on both its UV absorbance at 264nm and knowledge of its molar extinction coefficient so as to create a stock solution that was then stored at −20°C and protected from light. Immediately before use, dilutions of the stock solution in RPMI 1640 without FBS were made. The maximal concen-

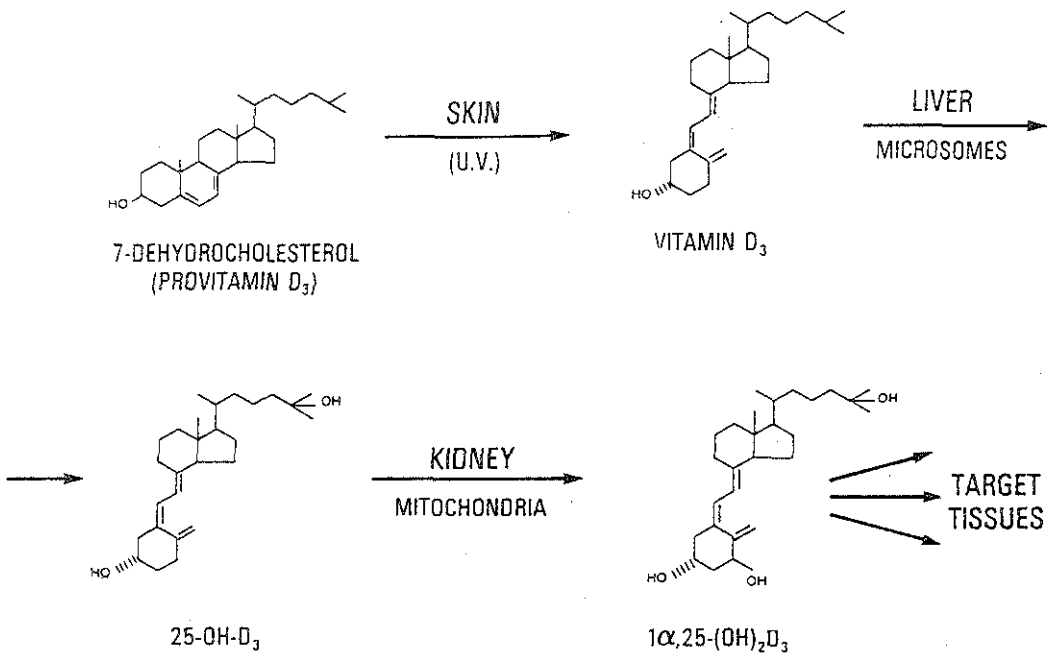

Fig. 1. Photobiosynthesis and functional metabolism of vitamin D_3.

tration of ethanol in the culture (0.1%) was not the factor that influences either cell growth or differentiation.

Growth inhibition

U937 cells were seeded at 10^5cells/ml in 96-well plates (Becton Dickson Labware, NJ) in RPM1 1640 supplemented with 10% FBS containing various concentration of the vitamin D_3 analog and incubated at 37° C. After 4 days, cultures were suspended with the number, and viable cells determined by trypan-blue were counted. Cell viabilty in both controlled and treated cells were similar (>90%). Results were represented as the means of triplicate culture. Inhibition of growth by the vitamin D_3 analog was calculated by the equation : % inhibition=1 − (cell count of treated culture)/(cell count of control culture).

Colony formation assay in soft agar

Cells were cultured in six-well culture dishes (Becton Dickson Labware, NJ) in a two-layer soft agar system as described in previous study[10]. The underlayer contained 0.5% agar and the upper layer contained 0.3% agar (Difco Laboratories, Inc., Detroit, MI). Compound and bone marrow cells were mixed into the underlayers and upperlayers, respectively. Cell conc-

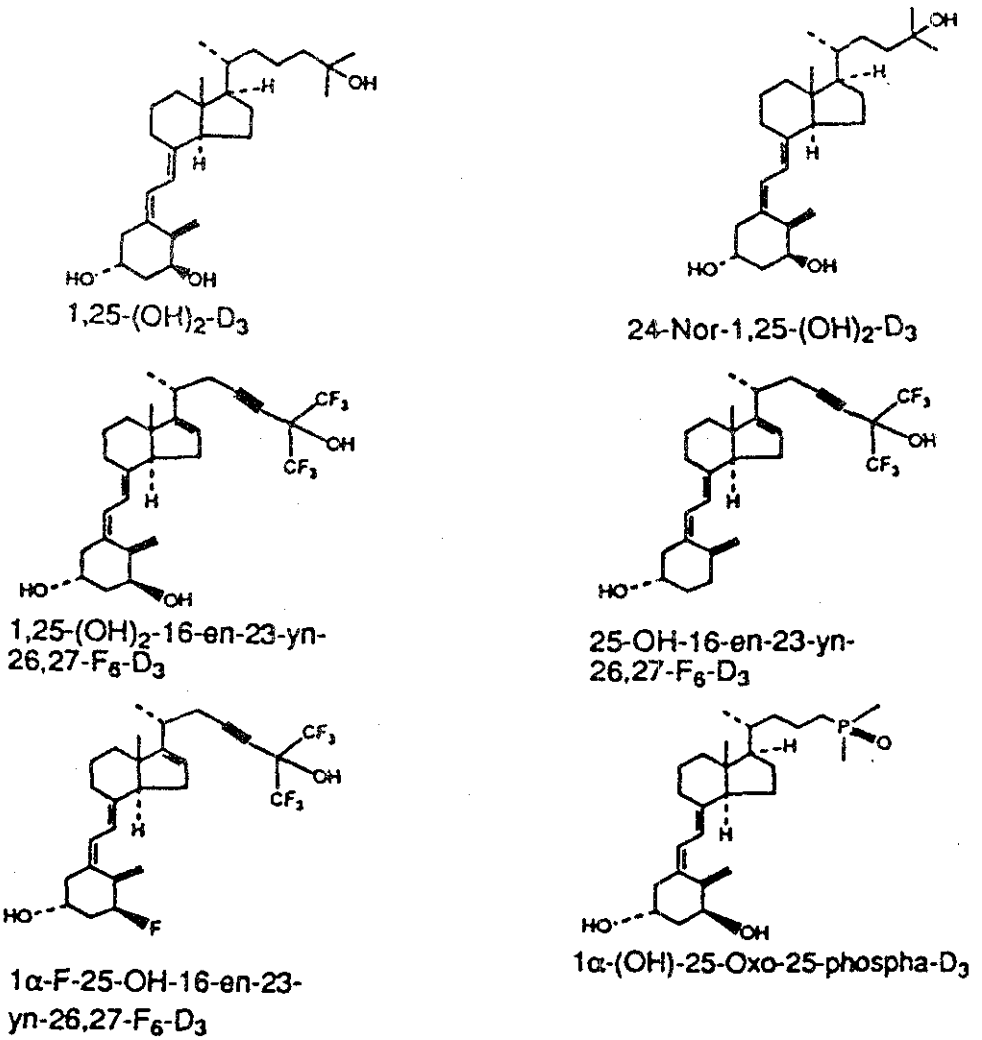

Fig. 2. Structural formular of novel 1.25(OH)2D3 analogs developed and 1,25 (OH)2-16ene-23yne-26yne-26,27-F6-D3.

entrations were 2×10^5 per plate. Recombinant granulocyte-monocyte colony-stimulating factor (GM-CSF, 200pm) was added to the underlayer as a source of CSF (generous gift of S. Clark, Genetics Institute, Boston, MA). Cultures were placed in a humidified atmosphere, 5% CO_2 at 37° C for 12 days. All experiments were done using triplicate plates per experimental point. Control plates with only CSF were performed for each experiment and colonies(>40 cells) were scored with an inverted microscope.

Induction of differentiation studies

Induction of differentiation of U937 cells was measured by morphology, adherence to plastic, and histochemistry using α-naphthyl acetate esterase (non-specific esterase [NSE]). The cells were grown in RPMI 1640 with 10% FBS containing various concentration of the 1,25 $(OH)_2D_3$ analog for 4 days in humidified 5.0% CO_2 atmosphere at 37° C. The cells were washed in PBS and cytocentrifuged. Slides were fixed for 30 seconds at room temperature in citrate-acetone-formaldehyde (25 : 65 : 8) solution, and were stained with a solution of 1ml fast blue BB base solution, 1ml sodium nitrate solution, 40ml prewarmed deionized water, 5ml trizmal pH 7.6 and 1ml naphthyl acetate solution for 30min at 37° C, protected from light. After staining the slides with hematoxilin solution for 2min, we checked them with a light microscope.

To examine the morphological change according to differentiation, Giemsa staining was done on the cytospin slide preparations.

RNA isolation and Northern blot

Total RNA of U937 cells was extracted by the hot phenol method and RNA blotting was basically performed as described by Maniatis *et al.*[19]. RNA samples were denatured at 65° C for 10min, size-separated by 1% agarose formaldehyde gel and transferred to a nylon membrane (Hybond N⁺, Amersham Co.). Filters were dried on the UV transmitter for 5min, and then prehybridized for 16~24h. Probes were the second exon (Pst I -Pst I fragment) of c-*myc*[20] and 3' nontranslated region of β-actin[21]. When used as probes, the DNA inserts were oligolabelled (random primer method) as described in the previous study[22]. Hybridization with [^{32}P]-oligolabelled c-*myc* and β-actin DNA was performed at 42° C for 24h in 50% formamide, 2× SSC (1×=150mM sodium chloride, 15 mM sodium citrate), 5× Denharts, 0.1% SDS, 10% dextran sulfate and 100μg/ml salmon sperm DNA. Filter was washed 3 times at a stringency of 3×. SSC, 0.1% SDS at 37° C for 1h, and washed again twice in 0.1×. SSC, 0.1% SDS at 50° C for 1h. Blots were exposed for 24~48h at −70° C on Kodak XAR-5 film. Modulation in levels of c-*myc* RNA was quantified by initial standardization to the amount of β-actin-specific transcript. The relative density of β-actin-specific and c-*myc*-specific transcript in the different lanes was first determined by laser densitometry using multiple exposures of the blot, and the ratio of c-*myc*/β-actin in the control lane was assigned to be the baseline level. The density in the experimental lane was calculated by multiplying the ratio of density of (c-*myc*/β-actin) transcript by the reciprocal of the ratio of the baseline level[23].

RESULTS AND DISCUSSION

Effect of vitamin D_3 analog on U937 cell proliferation

U937 cells proliferated with a doubling time of ~27h. At an initial culture density of ~1×10^5 cells/ml, maximal cell density was attained within 4~5 days. In suspension culture, the cells had a pleomorphic appearance with numerous projections. When maximum cell density was reached and exhausion of nutrient occurred, the cell rounded up and remained viable for 1~2 days before undergoing rapid cell death. 1,25$(OH)_2$-16ene-23yne-26,27-F_6-D_3 effected a dose-dependent inhibition of U937 cell proliferation as determined by cell count after 4 days in the culture (Table 1). With addition of 10^{-7}M and 10^{-8}M analog, the cell growth of U937 decreased to 87% and 62% of the control value after 4 days, respectively.

Effect of vitamin D_3 analog on clonal proliferation of normal myeloidcolony-forming cells

We investigated the effect of vitamin D_3 analog on the clonal growth of normal human myeloid stem ce-

lls which are known as granulocyte-macrophage colony forming cells (GM-CFC) grown in the presence of GM-CSF. Vitamin D_3 analog ideally should not inhibit normal growth of myeloid stem cells if they are used clinically. 1,25(OH)2-16ene-23yne-26,27-F_6-D_3 was not inhibited GM-CFC from bone marrow of normal individuals (Table 2). In fact this analog slightly stimulated the clonal myeloid growth.

Induction of differentiation by 1,25(OH)2-16ene-23yne-26,27-F_6-D_3

William *et al.*[24] and Inge *et al.*[25] demonstrated that U937 cells differentiated toward monocyte-macrophage like cells when cultured in the presence of 1,25 $(OH)_2D_3$. We also investigated the effect of 1,25(OH) 2-16ene-23yne-26,27-F_6-D_3 on U937 differentiation. Differentiation of cells to monocyte/macrophage characteristics was assessed by observation of cell morphology, cytochemical analysis of presence of α-napthyl acetate esterase activity and the appearance of adherent cells in the culture flasks. Fig. 3 shows the result of the nonspecific esterase (NSE) staining of U937 differentiated by 1,25 (OH)2-16ene-23yne-26, 27-F_6-D_3. The differentiated cells were intensely stained to dark red color. 1,25(OH)2-16ene-23yne-26, 27-F_6-D_3 induced U937 cells to differentiate to monocyte-macrophage like cells in a dose dependent fashion. As shown in Fig. 3, at concentration of 10^{-7}M and 10^{-8}M, the 1,25$(OH)_2D_3$ analog induced more than 90% U937 and 70% U937 to differentiate, respectively. In constrast, control plate not containing the compound had less than 7% macrophage like cells.

Induction of U937 cells differentiation by the 1,25 (OH)2-16ene-23yne-26,27-F_6-D_3 was accompained by striking morphologic change (Fig. 4). After four days in the culture, Giemsa staining of cytospin cell preparation revealed ruffling of their plasma membrane and increased cell size with a decrease in the nuclear-cytoplasmic ratio and loss of cytoplasmic basophilia. Together with the morphologic alterations of U937 cells induced by 1,25 (OH)2-16ene-23yne-26, 27-F_6-D_3, cell became adherent to flask at the end of culture. Table 3 is the result of the appearance of adherent cells in the culture flasks, and the number of adherent cells per culture increased in a dose dependent fashion. In addition, vitamin D_3 analog-induced cells developed pseudopodia and spread on glass surfaces (data not shown). These results suggest that U937 cells were morphologically and functionally differentiated to macrophage by a novel vitamin D_3 analog, 1,25 (OH)2-16ene-23yne-26,27-F_6-D_3, in a dose dependent.

Zhou *et al.*[18] showed that 1,25 (OH)2-16ene-23 yne-26,27-F_6-D_3, represented the most potent known 1,25$(OH)_2D_3$ analog as measured by the ability to induce differentiation and to inhibit clonal growth of HL-60 cells. They also showed that this compound

Table 1. Inhibition of U937 cell proliferation by 1,25(OH)2-16ene-23yne-26,27-F_6-D_3

1,25$(OH)_2D_3$ analog *concentration (M)*	Cell number (Day 4) *(cells/ml)*	% Inhibition
Control	5.6×10^7	0
10^{-11}	5.1×10^7	9
10^{-10}	4.4×10^7	21
10^{-9}	3.4×10^7	39
10^{-8}	2.1×10^7	62
10^{-7}	7.2×10^6	87

U937 cells were seeded at 1×10^5cells/ml in 96-well plates and incubated for 4 days with various concentration of 1,25 (OH)2-16ene-23yne-26,27-F_6-D_3. Viable cells (>90% in control and treated cultures) were counted using trypan blue exclusion and reported as the percentage of inhibition of cell growth. Inhibition of growth was calculated by the equation : % inhibition=1 - (cell concentration of treated culture)/cell concentration of control culture). Results are represented as the mean of *triplicate culture*

Table 2. Effect of 1,25$(OH)_2D_3$ analog on clonal proliferation of human myeloid colony-forming cells

1,25 $(OH)_2D_3$ analog *concentration (M)*	Colony numbers *(per plate)*
Control	101±21
10^{-11}	147±31
10^{-10}	154±32
10^{-9}	140±22
10^{-8}	121±26
10^{-7}	129±27

Cells were cultured in six-well culture dishes in a two layer soft agar system as described in the materials and methods. Bone marrow cells were seeded at 2×10^5cells/plate and placed in a humidified atmosphere, 5% CO_2 at 37° C for 12 days. All cultures were grown in the presence of maximally stimulating concentration of recombinant GM-CSF (200pmol/L) and colonies (>40cells) were scored with an inverted microscope. Values represent the mean±SD of 3 separate experiments with triplicate dishes

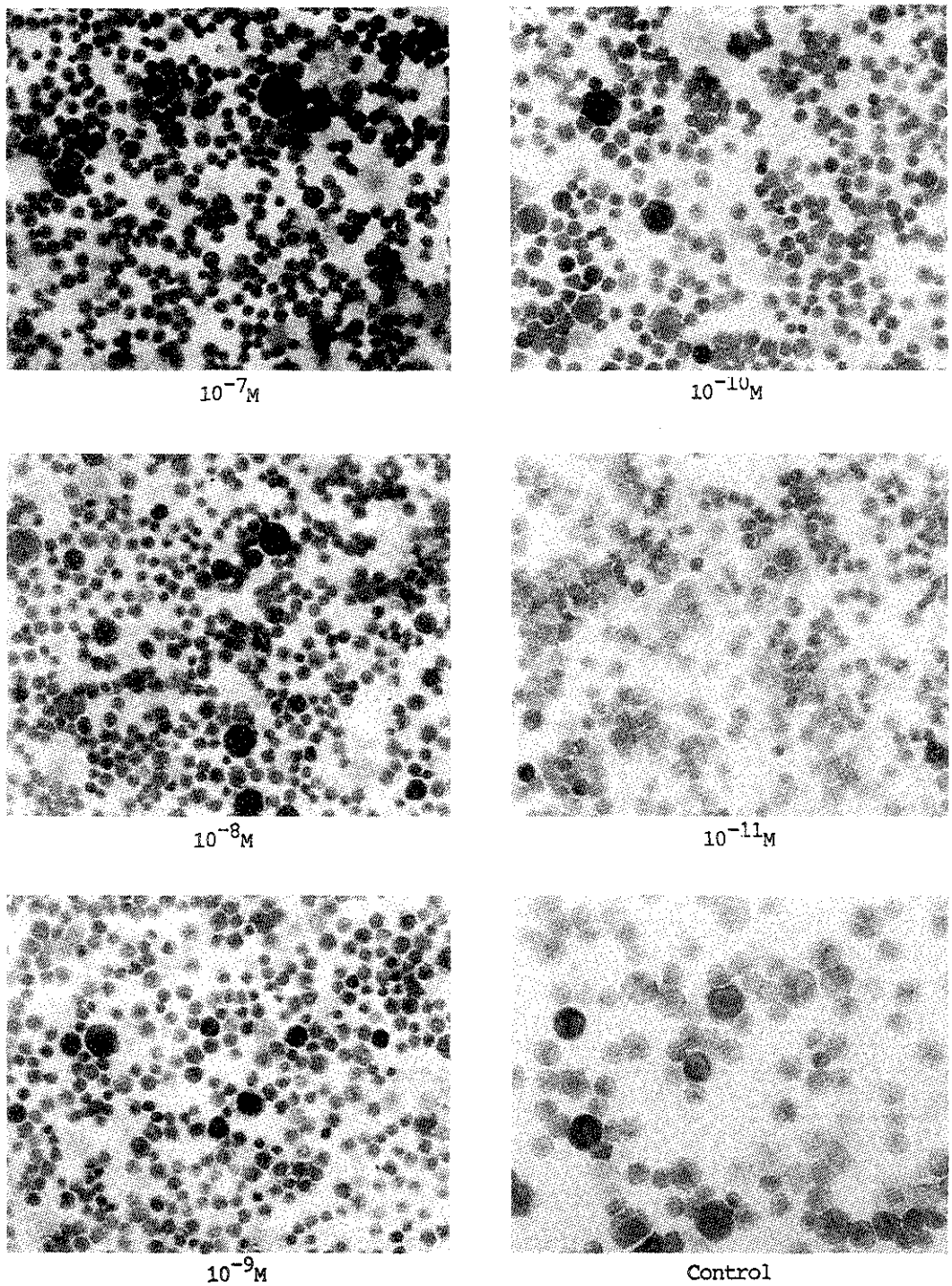

Fig. 3. Level of the nonspecific esterase in U937 cells differentiated by 1,25(OH)$_2$-16ene-23yne-26,27-F$_6$-D$_3$.
U937 cells (10^5cells/ml) in RPM1 1640 with 10% FBS were incubated for 4 days in the presence of 1,25(OH)$_2$-16ene-23yne-26,27-F$_6$-D$_3$(10^{-11}M~10^{-7}M). At the end of culture, cells were stained for nonspecific esterase (NSE) activity as a marker of cell differentiation. The differentiated cells were intensely stained to dark red color indicating mature macrophage, and the induction of differentiation of U937 cells by 1,25(OH)$_2$-16ene-23yne-26,27-F$_6$-D$_3$ was dose dependent. Control cells have less than 7% of NSE-positive cells (×200).

was 10-fold more potent than their previous reported most potent analog 1,25 (OH)$_2$-16ene-23yne-D$_3$ which has a double bond between carbons 16 and 17 and a triple bond between carbons 23 and 24 as compared to 1,25 (OH)$_2$D$_3$ and they observed that addition of six fluorines to 1,25(OH)$_2$D$_3$ increased potency. They explained that 1,25 (OH)$_2$-16ene-23yne-26,27-F$_6$-D$_3$ combines both of these features and the marked potency of this compound came from the structure feature. From the previous studies, we can also confer that the potency of 1,25(OH)$_2$-16 ene-23yne-26,27-F$_6$-D$_3$ in inhibiting cellular growth and inducing differentiation of U937 cells, comes from the structure feature of this compound.

On the other hand, the major toxicity of 1,25 (OH)$_2$-D$_3$ is hypercalcemia, which develops because of increased intestinal calcium absorption and mobilization of bone calcium. Zhou *et al.*[18] had previously observed the ICA (intestinal calcium absorption) and BCM (bone calcium mobilization) activities of 1,25 (OH)$_2$-16ene-23yne-26,27-F$_6$-D$_3$ and they had found that this compound was about 10 to 15 fold less potent than 1,25(OH)$_2$D$_3$ in ICA and BCM. This study shows that 1,25(OH)$_2$-16ene-23yne-26,27-F$_6$-

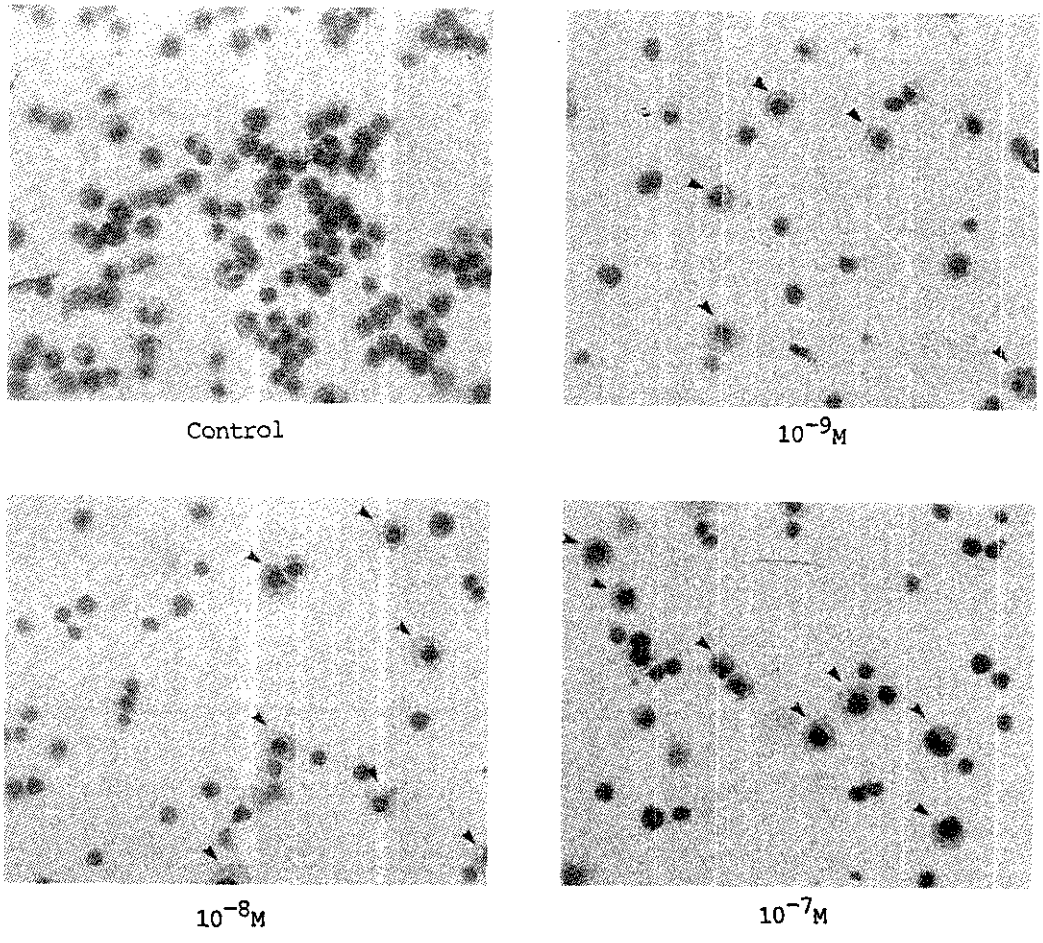

Fig. 4. Morphologic changes of the U937 cells induced by 1,25 (OH)$_2$-16ene-23yne-26,27-F$_6$-D$_3$ in a dose dependent fashion. Cells (10^5cells/ml) in RPMI 1640 with 10% FBS were incubated for 4 days in the presence of 1,25(OH)$_2$-16ene-23yne-26,27-F$_6$-D$_3$. Cells were washed in PBS, cytocentrifuged and fixed in methanol and stained with Giemsa for 10min. The smooth and small form of undifferentiated cells were transformed to the ruffled large form (indicated by arrow), and showed decreased N/C ratio in Giemsa staining (×200).

Table 3. Appearance of adherent U937 cells in the culture flasks

1,25(OH)$_2$D$_3$ analog concentration (M)	Adherence of U937cells 10^5cells per culture
Control	0.02±0.02
10^{-11}	0.11±0.11
10^{-10}	0.21±0.14
10^{-9}	0.64±0.32
10^{-8}	1.08±0.41
10^{-7}	1.56±0.64

U937 cells were seeded at 1×10^5cells/ml and incubated for 4 days in the presence of 1,25(OH)$_2$-16ene-23yne-26,27-F$_6$-D$_3$. At the end of culture adherent cells were counted. Values represent the mean±SD of 4~5 separate experiments

D$_3$ has little ability to cause either BCM or ICA.

Our present study and the result of the previous study[18] demonstrate that a novel 1,25(OH)$_2$-vitamin D$_3$ analog, 1,25(OH)$_2$-16ene-23yne-26,27-F$_6$-D$_3$, inhibits cellular growth and induces terminal differentiation of leukemic cells without causing hypercalcemia. This agent may be therefore medically useful compound for hematopoietic, skin and bone disorders. Furthermore, this analog will provide a tool to dissect the mechanism of action of vitamin D$_3$ seco-steroids in promoting cellular differentiation.

Effect of vitamin D$_3$ analog on c-*myc* mRNA accumulation

The U937 cells were cultured for 4 days in the presence of 1,25(OH)$_2$-16ene-23yne-26,27-F$_6$-D$_3$ and mRNA was harvested and examined for accumulation of levels of c-*myc* RNA by Northern blot (Fig. 5 Top). The blot was reprobed with β-actin to ensure equivalent amounts of the mRNA were present in each lane (Fig 5. Bottom). The viability of U937 cells was not affected at any of the experimental points, as determined by trypan blue exclusion. Exposure of 10^{-7}M 1,25(OH)$_2$-16ene-23yne-26,27-F$_6$-D$_3$ resulted in a ~60% reduction of c-*myc* mRNA levels (Top ; lane 2) compared with the untreated control sample (Top ; lane 1), as determined by densitometry readings. It has been suggested that c-*myc* RNA levels are a measure of the proliferative potential of leukemic cells[26,27], and a decrease of c-*myc* expression has been reported during induced differentiation of HL-60 and U937 cells[27~29]. Our results on the vitamin D$_3$ analog induced reduction of c-*myc* expression in U937 cells agreed with the other reports, and the above finding clearly supports that a novel vitamin D$_3$ analog, 1,25(OH)$_2$-16ene-23yne-26,27-F$_6$-D$_3$, may act as a differentiation-inducing factor to U937 cells.

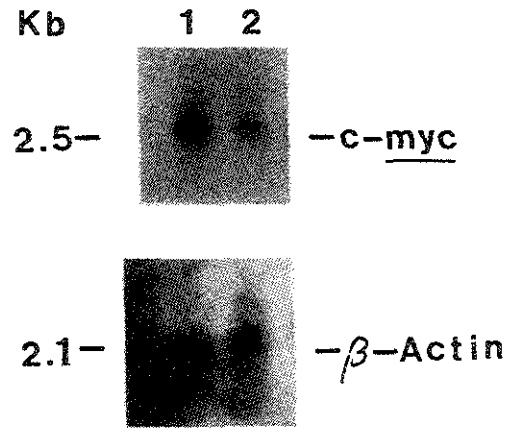

Fig. 5. Regulation of c-*myc* mRNA level in U937 cells by 1,25(OH)$_2$-16ene-23yne-26,27-F$_6$-D$_3$.
Lane 1, control (no compound) ; lane 2, 10^{-7}M. Top : RNA blot of hybridization with a DNA probe for c-*myc* (exonII, Pst-Pst). A single band could be detected at 2.5Kb which is consistent with c-*myc* mRNA. Bottom : the blot was reprobed with β-actin to ensure equivalent amounts of RNA were present in each lane. Each lane contains 20μg cytoplasmic RNA.

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인체 Histiocytic Lymphoma Cell Line U937의 증식 및 분화에 대한 새로운 1,25(OH)$_2$D$_3$ 유도체의 효과에 관한 연구

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요 약

인체 histiocytic lymphoma cell line U937의 증식억제 및 분화촉진 효과에 대한 새로운 vitamin D$_3$ 유도체인 1,25(OH)$_2$-16ene-23yne-26,27-F$_6$-D$_3$의 영향을 관찰하였다. 10^{-7}M 및 10^{-8}M 농도에서 이 화합물은 U937 cells의 증식을 각각 87% 및 62%까지 억제시켰다. 분화촉진 효과에 관한 연구에서는 세포의 morphology 변화와 세포분화의 marker로써 이용되는 nonspecific esterase (NSE) activity 및 배양기 세포의 adherence ability를 검토한 결과 이화합물은 U937 cells을 macrophage으로 분화시킴이 확인되었다. 또한 10^{-7}M 농도에서 이화합물은 U937 cells의 c-*myc* mRNA expression을 60% 정도 감소시킴이 확인되어 1,25(OH)$_2$-16ene-23yne-26,27-F$_6$-D$_3$는 U937 cells에 대해 분화를 유도하는 factor임을 본 연구 결과 알 수 있다.