Modification of $1\alpha,25$ -dihydroxyvitamin D_3 metabolism by introduction of 26,26,26,27,27,27-hexafluoro atoms in human promyelocytic leukaemia (HL-60) cells: isolation and identification of a novel bioactive metabolite, 26,26,26,27,27,27-hexafluoro- $1\alpha,23(S),25$ -trihydroxyvitamin D_3

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To study whether the introduction of fluoro atoms into C-26 and C-27 positions on the $1\alpha,25$ -dihydroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$] molecule could affect metabolism in human promyelocytic leukaemia (HL-60) cells, we compared the metabolism of 26,26,26,27,27,27-hexafluoro- $1\alpha,25$ -dihydroxyvitamin D_3 [26,27- F_6 - $1\alpha,25(OH)_2D_3$] and $1\alpha,25(OH)_2D_3$ in HL-60 cells. 26,27- F_6 - $1\alpha,25(OH)_2D_3$ was mainly converted into a new bioactive metabolite, 26,26,26,27,27,27-hexafluoro- $1\alpha,23(S),25$ -trihydroxyvitamin D_3 [26,27- F_6 - $1\alpha,23(S),25(OH)_3D_3$], but not into 26,26,26,27,27,27-hexafluoro- $1\alpha,24(R),25$ -trihydroxyvitamin D_3 [26,27- F_6 - $1\alpha,24(R),25(OH)_3D_3$] in HL-60 cells. 26,27- F_6 - $1\alpha,23(S),25(OH)_3D_3$ was identified by combina-

tions of h.p.l.c., u.v. spectroscopy and g.c.–mass spectrometry. Evidence is presented that 26,27- F_6 - $1\alpha,25(OH)_2D_3$ was metabolized to 26,27- F_6 - $1\alpha,23(S),25(OH)_3D_3$ by C-23 hydroxylation as a first step of the metabolism, and the 23-hydroxylated bioactive metabolite was accumulated in the cells, whereas $1\alpha,25(OH)_2D_3$ was initially deactivated and metabolized to $1\alpha,24(R),25(OH)_3D_3$ by C-24 hydroxylation through a side-chain oxidation pathway resulting in C_{23} – C_{24} cleavage, yielding 24,25,26,27-tetranor- $1\alpha,23(OH)_2D_3$ in HL-60 cells. These results show that 26,27- F_6 - $1\alpha,25(OH)_2D_3$ and $1\alpha,25(OH)_2D_3$ are metabolized by different metabolic pathways in HL-60 cells.

INTRODUCTION

$1\alpha,25$ -Dihydroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$], the hormonal form of vitamin D, induced differentiation of human promyelocytic leukaemia cells (HL-60) into monocyte–macrophages [1–3]. Up until now it has been reported that, among various fluorine-substituted analogues of $1\alpha,25(OH)_2D_3$, 26,26,26,27,27,27-hexafluoro- $1\alpha,25$ -dihydroxyvitamin D_3 [26,27- F_6 - $1\alpha,25(OH)_2D_3$] is about ten times more potent as an inducer compared with $1\alpha,25(OH)_2D_3$ in HL-60 cells [4,5] and other *in vitro* systems [6–9]. Also, this analogue is 5–10 times more potent than $1\alpha,25(OH)_2D_3$ in vitamin D-deficient rats [10] and chicks [11]. To elucidate the mechanism causing greater activity of 26,27- F_6 - $1\alpha,25(OH)_2D_3$ than $1\alpha,25(OH)_2D_3$, several investigators researched *in vivo* serum concentrations and clearances [12], membrane permeability of target-cell membrane [8,11], relative binding affinity to $1\alpha,25(OH)_2D_3$ receptor (VDR) [4,5,8] and the binding affinity of hormone–receptor complex to a DNA-affinity column [13] between two compounds. However, results for 26,27- F_6 - $1\alpha,25(OH)_2D_3$ were found to be nearly the same as those for $1\alpha,25(OH)_2D_3$. Such small differences in values do not account for the higher potency of the fluorinated compound. We compared metabolic pathways of the two compounds in HL-60

(human promyelocytic leukaemia) cells, because fluorine substitution of bioactive compounds often induces metabolic transformation and modulation of biological activities [14].

We report here that 26,27- F_6 - $1\alpha,25(OH)_2D_3$ was mainly metabolized to a new bioactive metabolite, 26,27- F_6 - $1\alpha,23(S),25(OH)_3D_3$ by C-23-hydroxylation as a first step of metabolism and the 23-hydroxylated metabolite accumulated in cells, causing greater differentiation-inducing activity of 26,27- F_6 - $1\alpha,25(OH)_2D_3$ than $1\alpha,25(OH)_2D_3$ in HL-60 cells.

A preliminary account of this work was given at the Eighth Workshop on Vitamin D, held in Paris, France, on 5–10 July 1991 [15].

MATERIALS AND METHODS

Materials

$1\alpha,25(OH)_2D_3$, 25-hydroxyvitamin D_3 [25-OH- D_3], $1\alpha,24(R),25$ -trihydroxyvitamin D_3 [$1\alpha,24(R),25(OH)_3D_3$], 24-oxo- $1\alpha,25$ -dihydroxyvitamin D_3 [24-oxo- $1\alpha,25(OH)_2D_3$], 24,25,26,27-tetranor- $1\alpha,23$ -dihydroxyvitamin D_3 [24,25,26,27-tetranor- $1\alpha,23(OH)_2D_3$], and [1β - 3H] $1\alpha,25(OH)_2D_3$ (18.2 Ci/mmol) were synthesized at Teijin Institute for Bio-medical Research, Tokyo, Japan. 26,26,26,27,27,27-Hexafluoro- $1\alpha,25$ -dihydroxyvitamin

Abbreviations used: $1\alpha,25(OH)_2D_3$, $1\alpha,25$ -dihydroxyvitamin D_3 ; HL-60 cells, human promyelocytic leukaemia cells; 26,27- F_6 - $1\alpha,25(OH)_2D_3$, 26,26,26,27,27,27-hexafluoro- $1\alpha,25$ -dihydroxyvitamin D_3 ; 26,27- F_6 - $1\alpha,23(S),25(OH)_3D_3$, 26,26,26,27,27,27-hexafluoro- $1\alpha,23(S),25$ -trihydroxyvitamin D_3 ; 26,27- F_6 - $1\alpha,24(R),25(OH)_3D_3$, 26,26,26,27,27,27-hexafluoro- $1\alpha,24(R),25$ -trihydroxyvitamin D_3 ; 25-OH- D_3 , 25-hydroxyvitamin D_3 ; $1\alpha,24(R),25(OH)_3D_3$, $1\alpha,24(R),25$ -trihydroxyvitamin D_3 ; 24-oxo- $1\alpha,25(OH)_2D_3$, 24-oxo- $1\alpha,25$ -dihydroxyvitamin D_3 ; 24,25,26,27-tetranor- $1\alpha,23(OH)_2D_3$, 24,25,26,27-tetranor- $1\alpha,23$ -dihydroxyvitamin D_3 ; 23-oxo-26,27- F_6 - $1\alpha,25(OH)_2D_3$, 23-oxo-26,26,26,27,27,27-hexafluoro- $1\alpha,25(OH)_2D_3$; 26,27- F_6 - $1\alpha,23(R),25(OH)_3D_3$, 26,26,26,27,27,27-hexafluoro- $1\alpha,23(R),25$ -trihydroxyvitamin D_3 ; 26,27- F_6 - $1\alpha,24(R),25(OH)_3D_3$, 26,26,26,27,27,27-hexafluoro- $1\alpha,24(R),25$ -trihydroxyvitamin D_3 ; FCS, fetal calf-serum; IMDM, Iscove's modified Dulbecco's medium; CM, conditioned medium; PBS(–), PBS without Ca^{2+} and Mg^{2+} ; Me₃Si, trimethylsilyl; e.i., electron impact; PMA, phorbol 12-myristate 13-acetate; VDR, $1\alpha,25(OH)_2D_3$ receptor; DBP, vitamin D-binding protein.

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D₃ [26,27-F₆-1 α ,25(OH)₂D₃] [16], 26,27-F₆-1 α ,23(S),25-(OH)₂D₃, 23-oxo-26,26,26,27,27,27-hexafluoro-1 α ,25-dihydroxyvitamin D₃ [23-oxo-26,27-F₆-1 α ,25(OH)₂D₃], 26,26,26,27,27,27-hexafluoro-1 α ,23(R),25-trihydroxyvitamin D₃ [26,27-F₆-1 α ,23(R),25(OH)₂D₃], 26,26,26,27,27,27-hexafluoro-1 α ,24(R),25-trihydroxyvitamin D₃ [26,27-F₆-1 α ,23(R),25(OH)₂D₃] and [1 β -³H]26,27-F₆-1 α ,25(OH)₂D₃ (18.8 Ci/mmol) were kindly provided by Sumitomo Pharmaceutical Co., Osaka, Japan. The following commercial materials were used: fetal-calf serum (FCS) from Whittaker M.A. Bioproducts Inc., Walkersville, MD; RPMI 1640 medium with L-glutamine, and Iscove's modified Dulbecco's medium (IMDM) with 2 mM L-glutamine and 25 mM Hepes buffer from Grand Island Biological Co., Grand Island, NY, U.S.A.

Cells and cell cultures

HL-60 cells were kindly supplied by Dr. S. Teitelbaum (Jewish Hospital, Washington University Medical Center, St. Louis, MO, U.S.A.). HL-60 cells were cultured in suspension in a 10% (v/v) serum-supplemented culture medium consisting of RPMI 1640 medium with L-glutamine, penicillin (100 units/ml), and streptomycin (100 μ g/ml) in a humidified CO₂/O₂ (1:19) atmosphere as described previously [17]. Metabolic experiments with 1 α ,25(OH)₂D₃ or 26,27-F₆-1 α ,25(OH)₂D₃ were performed by culturing HL-60 cells in a serum-free culture medium consisting of IMDM containing 2 mM L-glutamine, BSA (5 mg/ml), human transferrin (5 μ g/ml), penicillin (100 units/ml) and streptomycin (100 μ g/ml) for indicated times without changing the medium [18,19]. 1 α ,25(OH)₂D₃ or 26,27-F₆-1 α ,25(OH)₂D₃ dissolved in ethanol at < 0.2% concentration was added to cell cultures (final concns. 40 nM), and the same amount of vehicle (ethanol) was added to control cultures.

To prepare conditioned medium (CM), HL-60 cells were cultured in a 10% (v/v) serum-supplemented culture medium with 1 α ,25(OH)₂D₃ (40 nM) for 72 h at 37 °C. Medium was separated from cells by centrifugation at 700 g for 5 min at 4 °C, and used as conditioned medium.

Separation and purification of 26,27-F₆-1 α ,25(OH)₂D₃ metabolites by column chromatography and h.p.l.c.

HL-60 cells (1.6 $\times$ 10⁸ cells/400 ml) were incubated at 37 °C in a serum-free culture medium consisting of IMDM containing 2 mM L-glutamine, BSA (5 mg/ml) and human transferrin (5 μ g/ml) with 26,27-F₆-1 α ,25(OH)₂D₃ (40 nM) for times indicated. Cells were separated from medium by centrifugation at 700 g for 5 min at 4 °C, then washed with cold PBS without Ca²⁺ and Mg²⁺, pH 7.3 [PBS(–)] and sonicated four times (for 15 s each time) at 50 W with an ultrasonic cell disruptor (Heat systems–Ultrasonics, Farmingdale, NY, U.S.A.) in 2 ml of PBS(–). Tetrahydrofuran (1 ml) and ethyl acetate (4 ml) were added to the sonicated cells (2 ml), and the mixture was well vortexed-mixed. After centrifugation at 800 g for 5 min at 4 °C, the organic upper phase was withdrawn. The ethyl acetate extraction was repeated twice. The combined organic layer was evaporated with a rotary evaporator. The cell extracts were dissolved in 1 ml of propan-2-ol/n-hexane (1:24, v/v) and passed through a Sep-pak silica cartridge (Waters Associates, Milford, MA, U.S.A.). The Sep-pak cartridge was first eluted with 10 ml of propan-2-ol/n-hexane (1:24, v/v) and then with 8 ml of propan-2-ol/n-hexane (1:4, v/v). Metabolites eluted with propan-2-ol/n-hexane (1:4, v/v) were evaporated with a rotary evaporator for h.p.l.c. separation.

Culture medium (400 ml) was extracted with 2 vol. of chloroform/methanol (1:1, v/v), twice in separatory funnels, by using the method of Bligh and Dyer [20]. The lower chloroform phase was filtered through Whatman 1PS phase separators to remove water, evaporated with a rotary evaporator, and then the residue was dried by ethanol azeotrope. The chloroform extracts were chromatographed on a column (1.5 cm $\times$ 25 cm) of Sephadex LH-20 [21] and eluted, first with chloroform/n-hexane (13:7, v/v) (fractions 18–25; 4 ml/fraction tube), and then with chloroform/n-hexane/methanol (75:23:2, v/v) (fractions 26–45; 4 ml/fraction tube). 26,27-F₆-1 α ,25(OH)₂D₃ and its metabolite were eluted with fraction tubes 26–45 from the column, pooled, and concentrated.

Metabolite fractions from cells and medium were subjected to Shimadzu h.p.l.c. equipped with LC-6A pump and SPD-6A u.v. spectrophotometric detector on a straight-phase Zorbax Sil column (4.6 mm $\times$ 250 mm) with Shinwa-pack SI guard column (4 mm $\times$ 10 mm; Chromato Packings Center, Kyoto, Japan) eluted with n-hexane/propan-2-ol/dichloromethane (49:3:48, by vol.) at a flow rate of 1.5 ml/min. The effluent was continuously monitored by ultraviolet (u.v.) absorption at 264 nm and was collected in 1 min fractions using a programmable fraction collector (Advantec SF-2120). Absorbing peaks were separately collected, and further purified by h.p.l.c. rechromatography using the Zorbax Sil column (4.6 mm $\times$ 250 mm) eluted with propan-2-ol/n-hexane (3:17, by vol) at a flow rate of 1 ml/min. The sole 264 nm-absorbing peak in each fraction was pooled for structural identification.

In metabolic experiments involving [1 β -³H]26,27-F₆-1 α ,25(OH)₂D₃ or [1 β -³H]1 α ,25(OH)₂D₃, HL-60 cells (2 $\times$ 10⁶ cells/2 ml in a 35 mm-diameter dish) were incubated at 37 °C in a serum-free culture medium consisting of IMDM containing 2 mM L-glutamine, BSA (5 mg/ml) and human transferrin (5 μ g/ml) with [1 β -³H]26,27-F₆-1 α ,25(OH)₂D₃ (14 nM, 0.5 μ Ci) or with [1 β -³H]1 α ,25(OH)₂D₃ (14 nM, 0.5 μ Ci) for the times indicated. In each case a sample (2 ml) containing cells and culture medium was extracted with 2 vol. of chloroform/methanol (1:1, v/v) in a glass centrifuge tube [20]. After centrifugation at 1500 g for 10 min at 4 °C, the upper water/methanol layer and the lower chloroform layer were separated. The distribution of total radioactivity between the chloroform layer (containing various lipid-soluble metabolites) and the water/methanol layer (containing water-soluble metabolites) was estimated by measuring radioactivity in each layer.

Separation and purification of 1 α ,25(OH)₂D₃ metabolites by column chromatography and h.p.l.c.

HL-60 cells (1 $\times$ 10⁸ cells/100 ml) were incubated at 37 °C in a serum-free culture medium consisting of IMDM containing 2 mM L-glutamine, BSA (5 mg/ml) and human transferrin (5 μ g/ml) with 1 α ,25(OH)₂D₃ (40 nM) for times indicated. Cells were separated from medium by centrifugation at 700 g for 5 min at 4 °C. The cell and medium (100 ml) extracts with organic solvents were prepared and purified on a Sep-pak silica cartridge as described above. Metabolites eluted with propan-2-ol/n-hexane (1:4, v/v) from the Sep-pak cartridge were evaporated for h.p.l.c. separation. Metabolite samples from cells and medium were then subjected to h.p.l.c. on a straight-phase Zorbax Sil column (4.6 mm $\times$ 250 mm) with Shinwa-pack SI guard column (4 mm $\times$ 10 mm) eluted with 4% methanol in dichloromethane at a flow rate of 1 ml/min. The eluate was continuously monitored by u.v. absorption at 264 nm, and absorbing peaks were separately collected. The sole 264 nm-absorbing peak was pooled for structural identification.

In [1β - ^3H]1 α ,25(OH) $_2$ D $_3$ experiments, HL-60 cells (2×10^6 cells/2 ml of medium in a 35 mm-diameter dish) were incubated at 37 °C in a serum-free culture medium consisting of IMDM containing 2 mM L-glutamine, BSA (5 mg/ml) and human transferrin (5 $\mu\text{g}/\text{ml}$) with [1β - ^3H]1 α ,25(OH) $_2$ D $_3$ (14 nM, 0.5 μCi) for the times indicated. Sample solution (2 ml) containing cells and culture medium was extracted with 2 vol. of chloroform:methanol (1:1, v/v) in a glass centrifugal tube [20]. After centrifugation at 1500 g for 10 min at 4 °C, the lower chloroform layer was withdrawn and dried with a rotary evaporator. The chloroform extract was purified on the Sep-pak cartridge as described above. Metabolites eluted with propan-2-ol/n-hexane (1:4, v/v) from the Sep-pak cartridge were subjected to h.p.l.c. on the Zorbax Sil column (4.6 mm $\times$ 250 mm) with Shinwa-pak SI guard column (4 mm $\times$ 10 mm) eluted with 4% methanol in dichloromethane at a flow rate of 1 ml/min. Radioactivity in the effluent (1 ml) mixed with a 5 ml scintillation fluid was measured by a liquid-scintillation counter (Aloka-LSC 900).

U.v. spectroscopy

A Shimadzu SPD-M6A Photodiode Array UV-VIS Detector was used during metabolite-purification steps to select peaks possessing the u.v. spectral characteristics of the vitamin D 5,6-*cis*-triene chromophore (u.v._{max.} 265 nm, u.v._{min.} 228 nm, u.v._{max.}/u.v._{min.} = 1.8–2.0).

G.s.–m.s.

The metabolite (0.2–1 μg) or synthetic authentic standard was converted into trimethylsilyl (Me $_3$ Si) derivative by reacting with 40 μl of *NO*-bis(trimethylsilyl)acetamide (BSA, GL Sciences, Tokyo, Japan) for 30 min at room temperature in a Wheaton micro product V vial. The reaction mixture was dried up under N $_2$ gas and then dissolved in n-hexane. G.c.–m.s. was done using a HP-5890-II (g.c.) and a VG AutoSpec E (m.s.) with a 25 m long capillary column (DB-5), with injector at 285 °C and column temperature programmed from 90 to 270 °C at 10 °C/min and helium as carrier gas at a flow rate of 25 ml/min. For electron-impact (e.i.) m.s., ionization voltage was 70 eV.

RESULTS

HL-60 cell metabolism of 26,27-F $_6$ -1 α ,25(OH) $_2$ D $_3$

We examined the time course of 26,27-F $_6$ -1 α ,25(OH) $_2$ D $_3$ metabolites produced in HL-60 cells. Figure 1 shows that compounds in HL-60 cell fractions could be separated into two major peaks (a and b) by h.p.l.c. on a straight-phase Zorbax Sil column (4.6 mm $\times$ 250 mm) with Shinwa-pack SI guard column (4 mm $\times$ 10 mm) using n-hexane/propan-2-ol/dichloromethane (49/3/48, by vol.). Peaks a and b, with retention time of 27.2 min and 31.3 min respectively, were eluted at the same position as authentic 26,27-F $_6$ -1 α ,25(OH) $_2$ D $_3$ and 26,27-F $_6$ -1 α ,23(S),25(OH) $_3$ D $_3$. Also, authentic 26,27-F $_6$ -1 α ,23(R),25(OH) $_3$ D $_3$ and 26,27-F $_6$ -1 α ,24(R),25(OH) $_3$ D $_3$ had a retention time of 33.3 and 38.0 min respectively. The other peaks without u.v. spectral characteristics of the 5,6-*cis*-triene chromophore of vitamin D $_3$ were not characterized further in this study. Parent compound (peak a) was not metabolized in HL-60 cell fractions before 13 h exposure of HL-60 cells, but a major metabolite (peak b) apparently increased with decreasing parent compound after 1 day's exposure to 40 nM 26,27-F $_6$ -1 α ,

Figure 1 Time course of h.p.l.c. profiles of 26,27-F $_6$ -1 α ,25(OH) $_2$ D $_3$ metabolites produced in HL-60-cell fractions

HL-60 cells (1.6×10^6 cells/400 ml) were incubated at 37 °C in a serum-free culture medium consisting of IMDM containing 2 mM L-glutamine, BSA (5 mg/ml) and human transferrin (5 $\mu\text{g}/\text{ml}$) with 26,27-F $_6$ -1 α ,25(OH) $_2$ D $_3$ (40 nM) for (a) 13 h, (b) 1 day and (c) 3 days. Cells were separated from medium by centrifugation at 700 g for 5 min at 4 °C. Lipid extracts from cells or medium were purified on a Sep-pak cartridge or a Sephadex LH-20 column (1.5 cm $\times$ 25 cm) as described in the Materials and methods section. Metabolites were subjected to h.p.l.c. on a Zorbax Sil (4.6 mm $\times$ 250 mm) column with Shinwa-pack SI guard column (4 mm $\times$ 10 mm) using n-hexane/propan-2-ol/dichloromethane (49:3:48, by vol.) at a flow rate of 1.5 ml/min by monitoring u.v. absorption at 264 nm. The elution positions of authentic standards are shown by the arrows: a, 26,27-F $_6$ -1 α ,25(OH) $_2$ D $_3$; b, 26,27-F $_6$ -1 α ,23(S),25(OH) $_3$ D $_3$.

Figure 2 Time course of percentage changes in radioactivity distributed between lipid-soluble metabolites and water-soluble metabolites in HL-60 cells

HL-60 cells (2×10^6 cells/2 ml) were incubated at 37 °C in a serum-free culture medium with [1β - ^3H]26,27-F $_6$ -1 α ,25(OH) $_2$ D $_3$ (14 nM, 0.5 μCi ; ○, ●) or with [1β - ^3H]1 α ,25(OH) $_2$ D $_3$ (14 nM, 0.5 μCi ; △, ▲) for times indicated. The lipid extraction from samples (cells plus medium) and separation of the upper water/methanol layer from the lower chloroform layer were done as described in the Materials and methods section. The percentage of total radioactivity distributed between the chloroform layer containing various lipid-soluble metabolites (○, △) and the water/methanol layer containing water-soluble metabolites (●, ▲) was estimated by measuring radioactivity in each layer. Values represent means $\pm$ S.D. from duplicate cultures. In both 1β - ^3H -labelled compounds, the total recovered radioactivity calculated by summing lipid-soluble and water-soluble radioactivity remained almost constant (about 96%) at all time points, showing that the A-ring 1β - ^3H label was not lost to volatile products during extraction.

25(OH)₂D₃. In the medium fraction, only the parent compound (peak a) was detected up to 13 h exposure, but neither peak a nor peak b was detected after 1 day's exposure to 26,27-F₆-1 α ,25(OH)₂D₃ (40 nM) (results not shown). Figure 2 shows the time course of changes in the proportions of lipid-soluble or water-soluble metabolites present in extracts of HL-60 cells and incubation medium. In experiments with [1 β -³H]26,27-F₆-1 α ,25(OH)₂D₃, there was a slight increase in water-soluble radioactivity with time. Only 12% of the ³H radioactivity was extracted in water-soluble form after 72 h culture, suggesting little presence of water-soluble, ionized metabolites. Neither were other predicted metabolites of 26,27-F₆-1 α ,23(S),25(OH)₃D₃, viz. 26,27-F₆-24,25,26,27-tetranor-1 α ,23(OH)₂D₃ and calcitric acid, detected in cell or medium fraction. These results suggest that 26,27-F₆-1 α ,25(OH)₂D₃ was not metabolized in HL-60 cells until after 13 h exposure, and was mainly metabolized in HL-60 cells to 26,27-F₆-1 α ,23(S),25(OH)₃D₃ after 1 day's exposure to 26,27-F₆-1 α ,25(OH)₂D₃ (40 nM).

Identification of 26,27-F₆-1 α ,23(S),25(OH)₃D₃

The main peak-b component was further purified to homogeneity by h.p.l.c. on the Zorbax Sil column eluted with propan-2-ol/n-hexane (3:17, by vol). The peak-b component co-chromatographed with authentic 26,27-F₆-1 α ,23(S),25(OH)₃D₃, but did not co-chromatograph with the 23(R) epimer, 26,27-F₆-1 α ,23(R),25(OH)₃D₃ (Table 1).

Figure 3 shows u.v. absorption spectrum of the isolated peak-b metabolite. This metabolite displays u.v. spectral characteristics of the vitamin D₃ *cis*-triene chromophore with u.v._{max} 264 nm and u.v._{min} 227 nm.

Figure 4(a) shows gas-chromatogram of the trimethylsilyl ether (Me₃SiO) derivative of peak-b metabolite. The peak-b metabolite was separated into two peaks, a *pyro* form (retention time, 22.47 min) and an *isopyro* form (retention time, 24.34 min), by g.c. on 25 m long capillary column (DB-5), with temperature programmed from 90 to 270 °C at 10 °C/min and helium as carrier gas at a flow rate of 25 ml/min. Figure 4(b) shows e.i. mass spectrum (electron energy at 70 eV) of the Me₃SiO derivative (retention time, 22.47 min) of peak-b metabolite. The diagnostic ions and structural assignment are as follows: *m/z* 828 (*M*⁺, the molecular ion), 813 (*M*⁺ - CH₃), 738 (*M*⁺ - Me₃SiOH), 648 (*M*⁺ - 2 × Me₃SiOH), 558 (*M*⁺ - 3 × Me₃SiOH), 431 (*M*⁺ - side chain), 355 (C₂₂-C₂₃ cleavage), 341 (431 - Me₃SiOH), and 251 (341 - Me₃SiOH). This mass spectrum is identical with that

Figure 3 U.v. absorption spectrum of putative 26,27-F₆-1 α ,23(S),25(OH)₃D₃ (peak-b metabolite)

The maximum was at 264 nm.

of the corresponding derivative of synthetic 26,27-F₆-1 α ,23(S),25(OH)₃D₃, but quite different in *m/z* 341 from that of the corresponding derivative of synthetic 26,27-F₆-1 α ,24(R),25(OH)₃D₃. The characteristic mass fragment ion for structural assignments as 26,27-F₆-1 α ,23(S),25(OH)₃D₃ was 355 (C₂₂-C₂₃ cleavage; relative intensity, about 35%), whereas that of 26,27-F₆-1 α ,24(R),25(OH)₃D₃ was 341 (C₂₃-C₂₄ cleavage; relative intensity, about 60%). On the basis of g.c.-m.s. and u.v. spectral data as well as h.p.l.c. data presented, the structure of the new metabolite (peak-b metabolite) from 26,27-F₆-1 α ,25(OH)₂D₃ in HL-60 cells was determined to be 26,27-F₆-1 α ,23(S),25(OH)₃D₃.

H-60-cell metabolism of 1 α ,25(OH)₂D₃

We examined the time course of [1 β -³H]1 α ,25(OH)₂D₃ metabolites produced by HL-60 cells (Figure 5). The parent radioactive compound ([1 β -³H]1 α ,25(OH)₂D₃, peak a) on a straight-phase Zorbax Sil column (4.6 mm × 250 mm) with Shimwa-pack SI guard column (4 mm × 10 mm) using 4% methanol in dichloromethane was not metabolized in HL-60 cells until 13 h exposure at 37 °C, but radioactive metabolites (peaks b, c and d) apparently appeared with decreasing peak a after 1 day's exposure of HL-60 cells to [1 β -³H]1 α ,25(OH)₂D₃ (14 nM, 0.5 μ Ci). Peaks d, c and b, with a retention time of 11.5, 18.9 and 24.1 min respectively, were eluted at the same position as authentic 24-oxo-1 α ,25(OH)₂D₃, 24,25,26,27-tetranor-1 α ,23(OH)₂D₃ and 1 α ,24(R),25(OH)₃D₃. In contrast with [1 β -³H]26,27-F₆-1 α ,25(OH)₂D₃ (14 nM; 0.5 μ Ci) metabolism, at a [1 β -³H]1 α ,25(OH)₂D₃ (0.5 μ Ci) concentration of 14 nM, there was a marked time-dependent increase in water-soluble radioactivity (Figure 2). The radioactivity of water-soluble metabolites increased to about 46% of the ³H extracted from HL-60 cells and medium after 72 h culture, suggesting the conversion of lipid-soluble metabolites into possible water-soluble acidic metabolites such as calcitric acid through C₂₃-C₂₄ cleavage in a side-chain oxidation pathway.

To identify metabolites (peak b and c metabolites) by g.c.-m.s., the compounds were prepared on a large scale from the medium fraction of HL-60 cells exposed to 1 α ,25(OH)₂D₃ (40 nM) for 3 days, and further separated by h.p.l.c. (Figure 6). Peaks a, c and b of the medium fraction, with a retention time of 12.4, 15.5 and 21.6 min respectively, were eluted at the same positions as authentic 1 α ,25(OH)₂D₃, 24,25,26,27-tetranor-1 α ,23(OH)₂D₃ and 1 α ,24(R),25(OH)₃D₃.

Table 1 Co-chromatography of HL-60-cell metabolites of 26,27-F₆-1 α ,25(OH)₂D₃ with authentic standards

Putative 26,27-F₆-1 α ,25(OH)₂D₃ metabolites (about 100 ng in 20 μ l of mobile phase) were subjected to h.p.l.c. on a Zorbax Sil (4.6 mm × 250 mm) column with Shinwa-pack SI guard column (4 mm × 10 mm) using n-hexane/propan-2-ol/dichloromethane (49:3:48, by vol.) at a flow rate of 1.5 ml/min by monitoring u.v. absorption at 264 nm. The retention time was compared with that of authentic metabolites. Abbreviation: N.D., not detected.

	Retention time (min)	
	Standards	HL-60-cell metabolites
26,27-F ₆ -1 α ,25(OH) ₂ D ₃ metabolite		
23-Oxo-26,27-F ₆ -1 α ,25(OH) ₂ D ₃	23.6	N.D.
26,27-F ₆ -1 α ,25(OH) ₂ D ₃	27.2	Peak a
26,27-F ₆ -1 α ,23(S),25(OH) ₃ D ₃	31.3	Peak b
26,27-F ₆ -1 α ,23(R),25(OH) ₃ D ₃	33.3	N.D.
26,27-F ₆ -1 α ,24(R),25(OH) ₃ D ₃	38.0	N.D.

Figure 4 G.C. (a) and e.i.m.s. (b) of the trimethylsilyl ether derivative of putative 26,27- F_6 - $1\alpha,23(S),25(OH)_3D_3$ (peak b metabolite)

The g.c./e.i.m.s. conditions used are described in the Materials and methods section.

Figure 7 shows e.i.m.s. spectrum (electron energy at 70 eV) of the Me_3SiO derivative of peak-c metabolite. The diagnostic ions and structural assignments are as follows: m/z 576 (M^+), 561 ($M^+ - CH_3$), 486 ($M^+ - Me_3SiOH$), 471 ($486 - CH_3$), 396 ($M^+ - 2 \times Me_3SiOH$), 381 ($396 - CH_3$), and 251 ($M^+ - \text{side chain} - 2 \times Me_3SiOH$). This mass spectrum is similar to that reported in the literature for 24,25,26,27-tetranor- $1\alpha,23(OH)_2D_3$ [22]. The mass spectrum of the Me_3SiO derivative of the peak-b metabolite shows m/z 720 (M^+) and major mass fragment signals at 705 ($M^+ - CH_3$), and 630 ($M^+ - Me_3SiOH$). This mass spectrum of the peak-b metabolite was quite similar to that of the corresponding derivative of synthetic $1\alpha,24(R),25(OH)_3D_3$ [23]. From these results, peak-b and -c metabolites were determined to be $1\alpha,24(R),25(OH)_3D_3$ and 24,25,26,27-tetranor- $1\alpha,23(OH)_2D_3$ respectively.

Because phorbol 12-myristate 13-acetate (PMA) (100 nM, 1 day) or $1\alpha,25(OH)_2D_3$ (40 nM, 3 days) induces differentiation of HL-60 cells into monocyte-macrophage-like cells [17,18], we compared abilities of cells differentiated by treatment with PMA or $1\alpha,25(OH)_2D_3$ to convert $[1\beta\text{-}^3H]1\alpha,25(OH)_2D_3$ into $[1\beta\text{-}^3H]1\alpha,24(R),25(OH)_3D_3$ by intact cell assay, i.e., $1\alpha,25(OH)_2D_3$ 24-hydroxylase activity. $[1\beta\text{-}^3H]1\alpha,25(OH)_2D_3$ (14 nM, 0.5 μCi) was not metabolized by treatment with CM or by exposure to HL-60 cells for 13 h at 37 °C (Figures 8a and 8b). Cells differentiated with $1\alpha,25(OH)_2D_3$ (40 nM, 3 days) converted $[1\beta\text{-}^3H]1\alpha,25(OH)_2D_3$ to other metabolites, i.e., $[1\beta\text{-}^3H]1\alpha,24(R),25(OH)_3D_3$ (a major metabolite), $[1\beta\text{-}^3H]24\text{-oxo-}1\alpha,25(OH)_2D_3$ (a minor metabolite) and $[1\beta\text{-}^3H]24,25,26,27\text{-tetranor-}1\alpha,23(OH)_2D_3$ (a minor metabolite) before 13 h at 37 °C, whereas cells differentiated with PMA (100 nM, 1 day) did not

Figure 5 Time course of h.p.l.c. profiles of $[1\beta\text{-}^3\text{H}]1\alpha,25(\text{OH})_2\text{D}_3$ metabolites produced by HL-60 cells

HL-60 cells (2×10^6 cells/2 ml) were incubated at 37°C in a serum-free culture medium with $[1\beta\text{-}^3\text{H}]1\alpha,25(\text{OH})_2\text{D}_3$ (14 nM, $0.5 \mu\text{Ci}$) for (a) 13 h, (b) 1 day and (c) 2 days. Lipid extracts from samples (cells plus medium) were purified on a Sep-pak silica cartridge as described in the Materials and methods section. The metabolites were separated by h.p.l.c. on a Zorbax Sil (4.6 mm $\times$ 250 mm) column with Shinwa-pack SI guard column (4 mm $\times$ 10 mm) using 4% methanol in dichloromethane at a flow rate of 1 ml/min. Effluent radioactivity (1 ml) was measured by a liquid-scintillation counter. The elution positions of the various standards of $1\alpha,25(\text{OH})_2\text{D}_3$ metabolites are shown by the arrows: a, $1\alpha,25(\text{OH})_2\text{D}_3$; b, $1\alpha,24(R),25(\text{OH})_3\text{D}_3$; c, 24,25,26,27-tetranor- $1\alpha,23(\text{OH})_2\text{D}_3$; d, 24-oxo- $1\alpha,25(\text{OH})_2\text{D}_3$.

markedly induce $[1\beta\text{-}^3\text{H}]1\alpha,25(\text{OH})_2\text{D}_3$ metabolism before 13 h exposure (Figures 8c and 8d). These results suggest that only after exposure to $1\alpha,25(\text{OH})_2\text{D}_3$ do HL-60 cells show induction of $1\alpha,25(\text{OH})_2\text{D}_3$ metabolism, but the induction of monocyte differentiation does not alter its metabolism.

DISCUSSION

The present work clearly shows the differences in metabolic fate between the natural hormone $1\alpha,25(\text{OH})_2\text{D}_3$ and 26,27- F_6 - $1\alpha,25(\text{OH})_2\text{D}_3$ in HL-60 cells. Based on our and others' work [22,24,25], we propose the following metabolic pathway, particularly in the side-chain metabolism of $1\alpha,25(\text{OH})_2\text{D}_3$. It is probably metabolized in sequence to the inactive metabolites: $1\alpha,24(R),25(\text{OH})_3\text{D}_3$ by C-24 hydroxylation as a first step, 24-oxo- $1\alpha,25(\text{OH})_2\text{D}_3$ by C-24 oxidation and 24-oxo- $1\alpha,23,25(\text{OH})_3\text{D}_3$ by C-23 hydroxylation through a side-chain oxidation pathway resulting in $\text{C}_{23}\text{--C}_{24}$ cleavage, yielding 24,25,26,27-tetranor- $1\alpha,23(\text{OH})_2\text{D}_3$ (a major side-chain-cleaved lipid-soluble metabolite) as a precursor to calcitroic acid (a major water-soluble metabolite) [26,27] in HL-60 cells. Formally, $1\alpha,23,24,25(\text{OH})_4\text{D}_3$ or 24-oxo- $1\alpha,23,25(\text{OH})_3\text{D}_3$ could be the immediate precursor of the 24,25,26,27-tetranor- $1\alpha,23(\text{OH})_2\text{D}_3$. However, we could not detect $1\alpha,23,24,25(\text{OH})_4\text{D}_3$ in HL-60 cells, but detected the C-24 oxidation metabolites such as $1\alpha,24(R),25(\text{OH})_3\text{D}_3$ and 24-oxo- $1\alpha,25(\text{OH})_2\text{D}_3$. Thus we support the notion that metabolism of $1\alpha,25(\text{OH})_2\text{D}_3$ in HL-60 cells proceeds by the C-24 metabolic pathway previously described in perfused kidney [22,26] and in osteoblastic cell line UMR-106 [25,27]. In this metabolic pathway it is essential for both C-24- and C-23-hydroxylation steps to precede $\text{C}_{23}\text{--C}_{24}$ cleavage, i.e., catabolism and de-activation of $1\alpha,25(\text{OH})_2\text{D}_3$ in HL-60 cells.

Figure 6 H.p.l.c. profiles of $1\alpha,25(\text{OH})_2\text{D}_3$ metabolites in HL-60 cells

HL-60 cells (1×10^6 cells/100 ml) were incubated in a serum-free culture medium with $1\alpha,25(\text{OH})_2\text{D}_3$ (40 nM) for 3 days at 37°C . Cells were separated from medium by centrifugation at $700 g$ for 5 min at 4°C . Lipid extracts from cells and medium were purified on a Sep-pak silica cartridge as described in the Materials and methods section. Metabolites were separated by h.p.l.c. on a Zorbax Sil (4.6 mm $\times$ 250 mm) column with Shinwa-pack SI guard column (4 mm $\times$ 10 mm) using 4% methanol in dichloromethane at a flow rate 1 ml/min by monitoring u.v. absorption at 264 nm by a Photodiode Array UV-VIS Detector. To select peaks possessing the u.v. spectral characteristics of the vitamin D 5,6-*cis*-triene chromophore, each peak was re-analysed by scanning the full u.v. spectra (200–350 nm) using this detector. (a) Cell fraction and (b) medium fraction. Elution positions of the various standards of $1\alpha,25(\text{OH})_2\text{D}_3$ metabolites are shown by the arrows: a, $1\alpha,25(\text{OH})_2\text{D}_3$; b, $1\alpha,24(R),25(\text{OH})_3\text{D}_3$; c, 24,25,26,27-tetranor- $1\alpha,23(\text{OH})_2\text{D}_3$. The asterisk denotes vitamin D_3 *cis*-triene chromophore-free peak.

$1\alpha,25(\text{OH})_2\text{D}_3$ -dependent 25-OH- D_3 and $1\alpha,25(\text{OH})_2\text{D}_3$ 24 hydroxylases have been characterized in HL-60 cells [28,29]. Our results using intact-cell assays also show that the metabolic enzymes involved in the steps from $1\alpha,25(\text{OH})_2\text{D}_3$ to 24,25,26,27-tetranor- $1\alpha,23(\text{OH})_2\text{D}_3$ in HL-60 cells are probably $1\alpha,25(\text{OH})_2\text{D}_3$ -inducible. However, metabolism of $1\alpha,25(\text{OH})_2\text{D}_3$ is not obviously changed after induction of monocyte differentiation of HL-60 cells by this hormone.

We provide conclusive evidence that introduction of trifluoro atoms to the C-26 and C-27 positions on the $1\alpha,25(\text{OH})_2\text{D}_3$ molecule causes metabolic modification $1\alpha,25(\text{OH})_2\text{D}_3$ metabolism in HL-60 cells. First, 26,27- F_6 - $1\alpha,25(\text{OH})_2\text{D}_3$ is neither hydroxylated on C-23(R) nor C-24(R), but on C-23(S) as a first metabolic step in HL-60 cells, yielding a bioactive metabolite, 26,27- F_6 - $1\alpha,23(S),25(\text{OH})_3\text{D}_3$ [5]. This suggests that addition of 26,27-hexafluoro atoms to $1\alpha,25(\text{OH})_2\text{D}_3$ molecule may hinder sterically the access of 24-hydroxylase to 26,27- F_6 - $1\alpha,25(\text{OH})_2\text{D}_3$. Secondly, the 23-hydroxylated bioactive metabolite 26,27- F_6 - $1\alpha,23(S),25(\text{OH})_3\text{D}_3$ mainly accumulates in cells without undergoing further catabolism, judging from its scant conversion into water-soluble metabolites. This phenomenon may be explained by C-23 hydroxylation and no $\text{C}_{23}\text{--C}_{24}$ cleavage of the side chain of 26,27- F_6 - $1\alpha,25(\text{OH})_2\text{D}_3$, causing the 23-hydroxylated metabolite to accumulate in the cells, owing to blocked C-24 hydroxylation due to trifluorination at C-26 and C-27 of the $1\alpha,25(\text{OH})_2\text{D}_3$ molecule. We also presume that the C-23(S)-hydroxylated meta-

Figure 7 E.I.m.s. of Me_3SiO derivative of putative 24,25,26,27-tetranor- $1\alpha,23(OH)_2D_3$ (peak c metabolite)

The e.i.m.s. conditions used are described in the Materials and methods section

Figure 8 H.p.l.c. profiles of $[1\beta\text{-}^3H]1\alpha,25(OH)_2D_3$ metabolites produced by conditioned medium of HL-60 cells or by HL-60 cells differentiated with various reagents

(a) $[1\beta\text{-}^3H]1\alpha,25(OH)_2D_3$ (14 nM, 0.5 μCi) were incubated with CM for 13 h at 37 °C. CM was made as described in the Materials and methods section. (b) HL-60 cells (2×10^6 cells/2 ml) were incubated in a serum-free culture medium with $[1\beta\text{-}^3H]1\alpha,25(OH)_2D_3$ (14 nM, 0.5 μCi) for 13 h at 37 °C. (c) HL-60 cells (2×10^6 cells/2 ml) induced by $1\alpha,25(OH)_2D_3$ (40 nM, 3 days) were incubated in a serum-free culture medium with $[1\beta\text{-}^3H]1\alpha,25(OH)_2D_3$ (14 nM, 0.5 μCi) for 13 h at 37 °C. (d) HL-60 cells (2×10^6 cells/2 ml) induced by PMA (100 nM, 1 day) were incubated in a serum-free culture medium with $[1\beta\text{-}^3H]1\alpha,25(OH)_2D_3$ (14 nM, 0.5 μCi) for 13 h at 37 °C. The lipid extracts from samples (cells plus medium) were purified on a Sep-pak silica cartridge as described in the Materials and methods section. Metabolites were separated by h.p.l.c. on a Zorbax Sil (4.6 mm $\times$ 250 mm) column with Shinwa-pack SI guard column (4 mm $\times$ 10 mm) using 4% methanol in dichloromethane at a flow rate of 1 ml/min. Effluent radioactivity (1 ml) was measured by a liquid-scintillation counter. Elution positions of the various standards of $1\alpha,25(OH)_2D_3$ metabolites are shown by the arrowheads: a, $1\alpha,25(OH)_2D_3$; b, $1\alpha,24(R),25(OH)_3D_3$; c, 24,25,26,27-tetranor- $1\alpha,23(OH)_2D_3$; d, 24-oxo- $1\alpha,25(OH)_2D_3$.

bolite bound relatively strongly to VDR in HL-60 cells, because 26,27- F_6 - $1\alpha,23(S),25(OH)_3D_3$ possesses a relatively high percentage of binding affinity (about 30 %) for the VDR of HL-60 cells [5]. *In vivo* experiments by Nagata et al. [12] also showed that considerable amounts of 26,27- F_6 - $1\alpha,23(S),25(OH)_3D_3$ were detected in small intestine after administration of $[1\beta\text{-}^3H]26,27-F_6$ - $1\alpha,25(OH)_2D_3$ to vitamin D-deficient rats, but were not detected in rat serum. Thirdly, the lactone ring between C-23 and C-26 positions on the $1\alpha,25(OH)_2D_3$ molecule [30,31] cannot be formed from 26,27- F_6 - $1\alpha,25(OH)_2D_3$ in HL-60 cells, perhaps due to blocked C-26 hydroxylation of its side chain by substitution of $-CF_3$ for $-CH_3$ at C-26 and C-27 of the $1\alpha,25(OH)_2D_3$ molecule. Thus our data on the metabolism of 26,27- F_6 - $1\alpha,25(OH)_2D_3$ support the concept that the higher potency of 26,27- F_6 - $1\alpha,25(OH)_2D_3$ is not the result of its longer half-life [10], but rather that its major metabolite, 26,27- F_6 - $1\alpha,23(S),25(OH)_3D_3$, is bioactive and accumulates in HL-60 cells.

We showed previously that the order of relative percentage binding affinity for vitamin D-binding protein (DBP) in FCS was $1\alpha,25(OH)_2D_3$ (100 %) $\gg$ 26,27- F_6 - $1\alpha,25(OH)_2D_3$ (5.1 %) $>$ 26,27- F_6 - $1\alpha,23(S),25(OH)_3D_3$ ($< 1\%$) [5]. These results show that DBP-binding affinity for 26,27- F_6 - $1\alpha,25(OH)_2D_3$ in serum is about 20 times less than for $1\alpha,25(OH)_2D_3$. Yet the relative binding affinity of 26,27- F_6 - $1\alpha,25(OH)_2D_3$ for the VDR of HL-60 cells was about the same as that of $1\alpha,25(OH)_2D_3$ [5]. Thus another reason for the higher activity of 26,27- F_6 - $1\alpha,25(OH)_2D_3$ compared with that of $1\alpha,25(OH)_2D_3$ *in vitro* is probably that this analogue binds much less to the DBP and is thus more available to cells than $1\alpha,25(OH)_2D_3$ in serum-supplemented culture [5,19,32,33].

Analysis of the catabolic process of $1\alpha,25(OH)_2D_3$ in HL-60 cells may be important in understanding the cell-differentiation mechanism induced by $1\alpha,25(OH)_2D_3$. Fluorinated $1\alpha,25(OH)_2D_3$ compounds, which induce metabolic modification and modulation of bioactivity, can be useful in unravelling the action mechanism and metabolism of $1\alpha,25(OH)_2D_3$.

In conclusion, $1\alpha,25(\text{OH})_2\text{D}_3$ and $26,27\text{-F}_6\text{-}1\alpha,25(\text{OH})_2\text{D}_3$ metabolize in HL-60 cells by different pathways. One of the most likely mechanisms causing greater differentiation-inducing activity of $26,27\text{-F}_6\text{-}1\alpha,25(\text{OH})_2\text{D}_3$ than of $1\alpha,25(\text{OH})_2\text{D}_3$ is probably that $26,27\text{-F}_6\text{-}1\alpha,25(\text{OH})_2\text{D}_3$ is metabolized to the bioactive $26,27\text{-F}_6\text{-}1\alpha,23(\text{S}),25(\text{OH})_3\text{D}_3$ in HL-60 cells, whereas $1\alpha,25(\text{OH})_2\text{D}_3$ is degraded to inactive metabolites, i.e. $1\alpha,24(\text{R}),25(\text{OH})_3\text{D}_3$, $24\text{-oxo-}1\alpha,25(\text{OH})_2\text{D}_3$ and $24,25,26,27\text{-tetranor-}1\alpha,23(\text{OH})_2\text{D}_3$.

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