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EFFECT OF SUBSTITUTING FLUORINE FOR HYDROGEN AT C-26 AND C-27 ON THE SIDE CHAIN OF 1,25-DIHYDROXYVITAMIN D_3

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Abstract—Previous reports have demonstrated that introduction of fluorine atoms at C-26 and C-27 of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) results in the potentiation of various aspects of some biological activities. The higher biological activities of 26,26,26,27,27,27-hexafluoro-1,25-dihydroxyvitamin D_3 (26,27-F_6-1,25-$(OH)_2D_3$) were accounted for in part by a decrease in metabolic inactivation via the 26- and 27-hydroxylation pathways. In addition to 26,27-F_6-1,25-$(OH)_2D_3$ not being hydroxylated in the 26 and 27 positions, it did not undergo 24-hydroxylation despite a significant induction by 26,27-F_6-1,25-$(OH)_2D_3$ of 24-hydroxylase activity in the HL-60 cell system. Another fluorinated vitamin D_3 analog, 26,26,26,27,27,27-hexafluoro-1α-hydroxyvitamin D_3 (26,27-F_6-1α-OH-D_3) may not undergo 25-hydroxylation as efficiently as 1α-OH-D_3 *in vivo* because a rise in serum 26,27-F_6-1,25-$(OH)_2D_3$ levels after injection of 26,27-F_6-1α-OH-D_3 was delayed significantly with a much smaller amplitude. Furthermore, 26,26,26,27,27,27-hexafluoro-1,23(*S*),25-trihydroxyvitamin D_3 retained full activity in the induction of HL-60 cell differentiation even after 23(*S*)-hydroxylation, in contrast to 1,23(*S*),25-$(OH)_3D_3$. These data suggested that substitution of fluorines for hydrogens at C-26 and at C-27 positions may result in alteration in chemical reactivity and/or conformation of C-23, C-24 and C-25 positions of the 1,25-$(OH)_2D_3$ molecule.

The hexafluoroinated vitamin D_3 analog, 26,26,26,27,27,27 - hexafluoro - 1,25 - dihydroxyvitamin D_3 (26,27-F_6-1,25-$(OH)_2D_3$‡), is several times more potent than 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) in curing rickets, and in elevating and maintaining plasma calcium and phosphorus levels [1]. 1,25-$(OH)_2D_3$ induces the human promyelocytic leukemia cell line, HL-60, to differentiate into monocytes/macrophages via a steroid–hormone receptor mechanism [2–7]. Furthermore, 1,25-$(OH)_2D_3$ induces 1,25-$(OH)_2D_3$-24-hydroxylase in HL-60 mitochondria in a dose-dependent manner [8] and an up-regulation of the 1,25-$(OH)_2D_3$ receptor [9], thus providing a relevant system for the investigation of the molecular mechanism of 1,25-$(OH)_2D_3$ action. As well as having a calcium mobilizing effect, 26,27-F_6-1,25-$(OH)_2D_3$ is about ten times as potent as 1,25-$(OH)_2D_3$ in the suppression of HL-60 cell proliferation and in the induction of cell differentiation [5]. Initially, the reason for the enhanced biological activities was accounted for by several mechanisms [10] including a decreased metabolic inactivation by way of 26- and 27-hydroxylation owing to the substituted fluoro groups at the 26- and 27-carbons [1]. However, in the present study, we demonstrate that substitution of fluorines for the hydrogens on C-26 and C-27 positions of 1,25-$(OH)_2D_3$ may block hydroxylation not only at C-26 and C-27 positions but also at C-24 and C-25 positions and that it may change the conformation of the C-23 position of the 1,25-$(OH)_2D_3$ molecule.

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‡ Abbreviations: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 1α-OH-D_3, 1α-hydroxyvitamin D_3; 1,23(*S*),25-$(OH)_3D_3$, 1,23(*S*),25-trihydroxyvitamin D_3; and 26,27-F_6-1,25 - $(OH)_2D_3$ 26,26,26,27,27,27 - hexafluoro - 1,25 - dihydroxyvitamin D_3.

MATERIALS AND METHODS

Materials. Radioactive 1,25-$(OH)_2$[26,27-^{3}H]D_3 (164.4 Ci/mmol) and [1β-^{3}H]26,27-F_6-1,25-$(OH)_2D_3$ was obtained from Dupont/New England Nuclear (Boston, MA) and from the Sumitomo Pharmaceutical Co. (Osaka, Japan), respectively. Non-radioactive 1,25-$(OH)_2D_3$ was a gift from the Chugai Pharmaceutical Co. (Tokyo, Japan). 26,27-F_6-1,25-$(OH)_2D_3$, 26,27-F_6-1α-hydroxyvitamin D_3 (26,27-F_6-1α-OH-D_3) and 26,27-F_6-1,23(*S*),25-trihydroxyvitamin D_3 (26,27-F_6-1,23(*S*),25-$(OH)_3D_3$) were obtained from the Sumitomo Pharmaceutical Co.

Cells and cell culture. HL-60 cells were provided by Dr. N. Negoro (First Department of Internal Medicine, Osaka City University Medical School). HL-60 cells were cultured as described previously [4, 5]. Briefly, HL-60 cells were cultured in RPMI 1640 medium (Flow Laboratories, Scotland, U.K.) containing 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY) in a humidified atmosphere of 5% CO_2 in air at 37°. Under these conditions, the doubling time was less than 48 hr. Cell differentiation was assessed by the nitroblue tetrazolium (NBT) reduction assay, as described previously [4, 5]. Cells

were counted by hemacytometer, and cell viability was determined by the trypan blue exclusion method.

Analyses of the metabolites of 1,25-$(OH)_2$[26,27-^{3}H]D_3 *or* [1β-^{3}H]26,27-F_6-1,25-$(OH)_2D_3$ *in HL-60 cells by HPLC.* HL-60 cells (5×10^4 cells/mL) were incubated with 10^{-7} M 1,25-$(OH)_2D_3$ and 10^{-9} M 26,27-F_6-1,25-$(OH)_2D_3$ for 24 and 72 hr, respectively. Then each group of cells was incubated with either 10 pmol of [1β-^{3}H]26,27-F_6-1,25-$(OH)_2D_3$ (345 dpm/pmol) or 10 pmol of 1,25-$(OH)_2$[26,27-^{3}H]D_3 (3000 dpm/pmol) for 24 hr, respectively. After the cells were washed twice with calcium^{2+} and magnesium^{2+}-free phosphate-buffered saline (PBS), 0.6 mL of 2:1 (v/v) methanol:chloroform was added to cell pellets, and sterols were extracted by the method of Bligh and Dyer [11] with a slight modification [12]. Prior to extraction, unlabeled 26,27-F_6-1,25-$(OH)_2D_3$ and 26,27-F_6-1,23(*S*),25-$(OH)_3D_3$ or unlabeled 1,25-$(OH)_2D_3$ and 1,24,25-$(OH)_3D_3$ were added to each tube as an internal standard for the determination of yield and for product authentication. The mixtures were kept on ice for 16 hr, and then the sterols were recovered from individual mixtures by transferring the supernatant to 1.5-mL polypropylene tubes (Eppendorf, Brinkmann Instruments, Westbury, NY) containing 0.2 mL of chloroform and 0.1 mL of water. The mixtures were shaken vigorously, and phase separation of the aqueous and organic layers was accomplished by centrifugation at 2000 *g* for 1 min. The chloroform layer was removed carefully with a 21-gauge needle, and the aqueous phase was reextracted by the addition of 0.4 mL of chloroform. After mixing and phase separation, the second chloroform extract was combined with the first. The samples were then dried under nitrogen and dissolved in 200 μL of toluene:ethanol (1:1 v/v) for storage. Extraction efficiency was usually greater than 90% as monitored by recovery of radioactivity.

Identification and determination of vitamin D_3 metabolites were performed with a Waters model ALC/GPC 204 (Waters Associates, Milford, MA) HPLC using a Zorbax-Sil column (0.46 × 25 cm, Dupont Instruments, Wilmington, DE) at a flow rate of 1.5 mL/min with 3.0% methanol:50% CH_2Cl_2:47% *n*-hexane for [1β-^{3}H]26,27-F_6-1,25-$(OH)_2D_3$ and at a flow rate of 2.0 mL/min with 5.5% methanol:20% CH_2Cl_2:74.5% *n*-hexane for 1,25-$(OH)_2$[26,27-^{3}H]D_3, as previously described [8, 11].

Determination of serum levels of 26,27-F_6-1,25-$(OH)_2D_3$ *and* 1,25-$(OH)_2D_3$. Serum levels of vitamin D analogs were determined as described previously [12]. Briefly, 1.0 mL of blood was shaken vigorously with 2.0 mL of ethyl acetate for 1 min and then centrifuged at 2000 *g* for 5 min. The supernatant was then dried under nitrogen, and the residue was suspended in 200 μL of ethanol. Next the sample was applied to a Sep-Pak silica column (Waters Associates) which had been prewashed with 5.0 mL of ethanol, 5.0 mL of *n*-hexane and finally with 5.0 mL of 4% isopropanol:96% *n*-hexane. After the column was washed with 10 mL of 4% isopropanol:96% *n*-hexane, it was treated with a mixture of 20% isopropanol:80% *n*-hexane. The first 8.0 mL were collected, in which the dihydroxy metabolites of vitamin D_3 were contained. The sample was dried under nitrogen and then applied to an HPLC system using a Radial-Pak cartridge column (8 mm × 10 cm, μPORASIL, Waters Associates) at a flow rate of 2.0 mL/min. The solvents used for the separation were 6% isoproterenol:94% *n*-hexane for 26,27-F_6-1,25-$(OH)_2D_3$ and 8% isoproterenol:92% *n*-hexane for 1,25-$(OH)_2D_3$, respectively. The fractions at which authentic 26,27-F_6-1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ migrated were collected and then evaporated for use in a competitive protein binding assay using chick intestinal cytosol (Yamasa Co. Ltd., Tokyo, Japan) as a 1,25-$(OH)_2D_3$ receptor source as previously described [12].

Measurement of the ability of HL-60 cells to hydroxylate the C-24 position of 1,25-$(OH)_2$[26,27-^{3}H]D_3. HL-60 cells were assayed for their ability to hydroxylate 1,25-$(OH)_2$[26,27-^{3}H]D_3 to form 1,24,25-$(OH)_3$ [26,27-^{3}H]D_3 by using the periodate-based method as described [13–15]. Briefly, after treatment with the indicated concentrations of 1,25-$(OH)_2D_3$ or 26,27-F_6-1,25-$(OH)_2D_3$ for 24 hr, cells were subjected to a vitamin D_3 "washout" procedure [16] to remove added vitamin D_3 compound that might compete with 1,25-$(OH)_2$[26,27-^{3}H]D_3 for the substrate on the 24-hydroxylase. The cells were then washed twice with RPMI 1640 medium containing 2% FBS. Finally, washed cells were resuspended at 1.0×10^7 cells/mL. An aliquot (0.2 mL) of the cell suspension was added to each test tube containing 10 pmol of nitrogen-dried radioactive substrate (1,25-$(OH)_2$[26,27-^{3}H]D_3, 4000 dpm/pmol) and incubated at 37° for 3 hr in a shaking water bath. The reaction was stopped by the addition of 0.3 mL of 0.1 N acetic acid, followed by the addition of 0.5 mL of saturated sodium periodate. The [^{3}H]acetone generated by periodate cleavage at the bond between C-24 and C-25 of 1,24,25-$(OH)_3$[26,27-^{3}H]D_3 was separated from 1,25-$(OH)_2$[26,27-^{3}H]D_3 by filtration through a 1-mL (C-18) solid-phase extraction cartridge (Supelco, Bellefonte, PA) and counted for radioactivity.

Calcium mobilization assays. Vitamin D deficiency was induced by the standard method [17] with slight modification [18]. Briefly, 7 days after insemination, pregnant dams were placed on a synthetic vitamin D-deficient diet that contained 0.45% of calcium and 0.3% of phosphorus (Clea Japan Co., Tokyo, Japan); they were then kept in the dark or under fluorescent light. After birth, male pups were fed the same diet for 3 weeks. Pups were then placed on the same vitamin D-deficient diet but containing only 0.02% calcium 3 days before the experiments. The animals were then divided into 3 groups and were given 500 pmol/100 g body weight of 1,25-$(OH)_2D_3$ or 5000 pmol/100 g body weight of 26,27-F_6-1α-OH-D_3 by a single intrajugular injection of vitamin D_3 compound dissolved in 95% ethanol (0.35 mL/kg body weight). Bone calcium mobilization was estimated by the rise in serum calcium of the vitamin D-deficient rats on the low calcium diet after a single injection of vitamin D_3 compound [19]. Serum calcium concentrations were measured by an automated technique, as described [20].

Statistical significance. Statistical analysis was done by Student's *t*-test. A level of $P < 0.05$ was regarded as statistically significant.

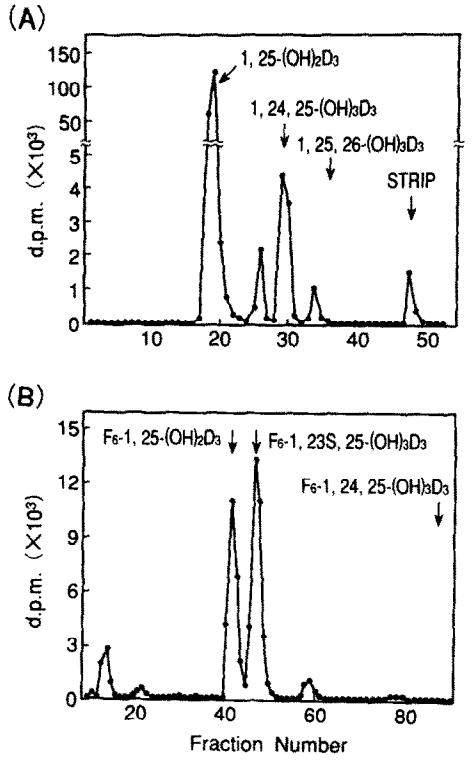

Fig. 1. Vitamin D_3 metabolites produced by the *in vitro* incubation of HL-60 cells with 1,25-$(OH)_2D_3$[26,27-^{3}H]D_3 or [1β-^{3}H]26,27-F_6-1,25-$(OH)_2D_3$. HL-60 cells were treated with 10^{-7} M 1,25-$(OH)_2D_3$ (A) and 10^{-9} M 26,27-F_6-1,25-$(OH)_2D_3$ (B) for 15 and 72 hr, respectively. Then each group of cells was incubated with either 10 pmol of 1,25-$(OH)_2$[26,27-^{3}H]D_3 (3000 dpm/pmol) (A) or 10 pmol of [1β-^{3}H]26,27-F_6-1,25-$(OH)_2D_3$ (345 dpm/pmol) (B) for 24 hr. After the cells were washed twice with calcium^{2+}- and magnesium^{2+}-free PBS, 0.6 mL of methanol:chloroform (2:1, v/v) was added to the cell pellets and sterols were extracted by the method of Bligh and Dyer [11] with a slight modification [13]. Then the sterols were subjected to straight-phase HPLC on a Zorbax-Sil column as described under Materials and Methods. The elution position of each vitamin D_3 compound was assessed with radioinert standards by measuring absorbance at 254 nm.

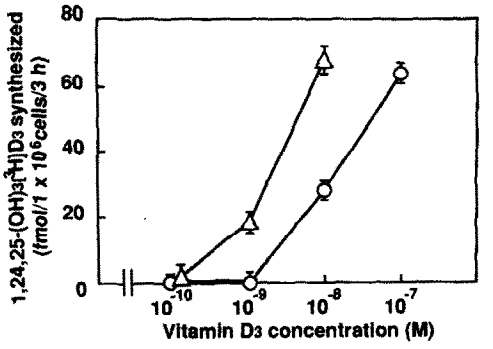

Fig. 2. Dose–response effect of 1,25-$(OH)_2D_3$ and 26,27-F_6-1,25-$(OH)_2D_3$ on the induction of HL-60 24-hydroxylase activity. HL-60 cells were treated with 10^{-10} to 10^{-7} M 1,25-$(OH)_2D_3$ (○) or 26,27-F_6-1,25-$(OH)_2D_3$ (△) for 24 hr. The cells (2.0×10^6 cells/tube) were incubated with 10 pmol of 1,25-$(OH)_2$[26,27-^{3}H]D_3 at 37° for 3 hr in a shaking water bath. The production of 1,24,25-$(OH)_3$[26,27-^{3}H]D_3 was determined by a periodate-based method as described [8, 13, 14]. Each point is the mean ± SD of three cultures.

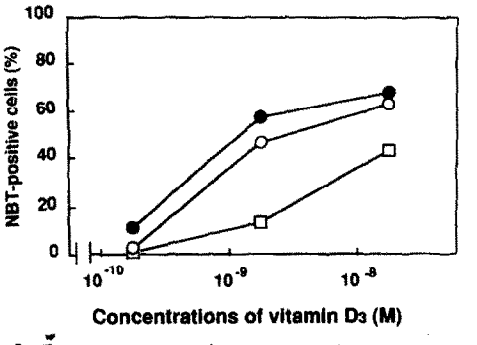

Fig. 3. Dose–response effect of induction of HL-60 cell differentiation by 1,25-$(OH)_2D_3$ and fluorinated vitamin D_3 analogs. HL-60 cells were incubated with each derivative of vitamin D_3 as described under Materials and Methods. Cell differentiation was assessed on day 3 by the NBT reduction assay [4, 5]. Data were highly reproducible. Key: (●) 26,27-F_6-1,25-$(OH)_2D_3$; (○) 26,27-F_6-1,23(*S*),25-$(OH)_3D_3$; and (□) 1,25-$(OH)_2D_3$.

RESULTS

Metabolism of 1,25-$(OH)_2$[26,27-^{3}H]D_3 *or* [1β-^{3}H]26,27-F_6-1,25-$(OH)_2D_3$ *in HL-60 cells.* We have reported that the major metabolite *in vivo* of 26,27-F_6-1,25-$(OH)_2D_3$ is 26,27-F_6-1,23(*S*),25-$(OH)_3D_3$, which is different from 1,25-$(OH)_2D_3$ in that its major metabolic pathway is known to be via the 24-hydroxylation pathway [18]. In the present study, we examined the metabolism of 26,27-F_6-1,25-$(OH)_2D_3$ along with that of 1,25-$(OH)_2D_3$ in the HL-60 cell system. The radioactive profile of the lipid extract derived from incubation of the cells with 1,25-$(OH)_2$[26,27-^{3}H]D_3 is shown in Fig. 1A. HL-60 cells synthesized a tritiated metabolite that comigrated with standard 1,24,25-$(OH)_3D_3$. This metabolite was identified as 1,24,25-$(OH)_3D_3$ because it comigrated on reverse-phase HPLC with authentic 1,24,25-$(OH)_3D_3$ and lost over 95% of its radioactivity by periodate treatment (data not shown). The formation of a peak was detected between 1,25-$(OH)_2D_3$ and 1,24,25-$(OH)_3D_3$, which was resistant to periodate treatment (data not shown). These results, taken collectively with a previous report of ours indicating that 1,25-$(OH)_2D_3$ induces 1,25-$(OH)_2D_3$-24- and 23(*S*)-hydroxylase activity in HL-60 mitochondria [8], seemed to indicate that this minor metabolite was 1,23(*S*),25-$(OH)_3D_3$. In contrast, 26,27-F_6-1,25-$(OH)_2D_3$ was metabolized exclusively to 26,27-F_6-1,23(*S*),25-$(OH)_3D_3$ as shown in Fig. 1B. These data may suggest that 26,27-F_6-1,25-$(OH)_2D_3$ does not undergo

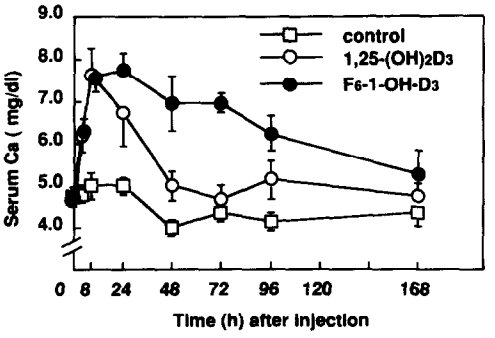

Fig. 4. Time course of the effects of 1,25-$(OH)_2D_3$ and 26,27-F_6-1α-OH-D_3 on serum calcium concentration in vitamin D-deficient rats after a single intrajugular injection of each vitamin D_3 compound. Six rats were administered 5000 pmol/100 g body weight of 26,27-F_6-1α-OH-D_3, 500 pmol/100 g body weight of 1,25-$(OH)_2D_3$, or vehicle. Blood samples were drawn serially from retroorbital venous plexus using hematocrit capillary tubes at the indicated times. All data are given as means ± SEM of six rats.

24-hydroxylation despite a significant induction of 24-hydroxylase activity. Another possibility is that introduction of fluorine atoms at the C-26 and C-27 positions of 1,25-$(OH)_2D_3$ may result in a qualitative change so that 26,27-F_6-1,25-$(OH)_2D_3$ fails to induce 24-hydroxylase activity in HL-60 mitochondria.

Effect of 1,25-$(OH)_2D_3$ *or* 26,27-F_6-1,25-$(OH)_2D_3$ *on the induction of cell activity to hydroxylate* C-24 *of* 1,25-$(OH)_2$[26,27-^{3}H]D_3. We have demonstrated recently that 1,25-$(OH)_2D_3$ specifically induces 1,25-$(OH)_2D_3$-24-hydroxylase activity in HL-60 mitochondria in a dose-dependent manner [8] and that quantification of 1,24,25-$(OH)_3$[26,27-^{3}H]D_3 produced by HL-60 cells as measured by our developed periodate-based method is in good correlation with that by the well-established HPLC method [8, 13]. After HL-60 cells had been treated in the presence of 5% FBS with various concentrations of 1,25-$(OH)_2D_3$ or 26,27-F_6-1,25-$(OH)_2D_3$ for 24 hr, the ability of the cells to hydroxylate the C-24 position of 1,25-$(OH)_2$[26,27-^{3}H]D_3 was determined. As shown in Fig. 2, 26,27-F_6-1,25-$(OH)_2D_3$ was approximately four times more potent than 1,25-$(OH)_2D_3$ in this action.

Biological activity of 26,27-F_6-1,23(S),25-$(OH)_3D_3$ *on the induction of cell differentiation.* Figure 3 shows the dose–response effect of fluorinated derivatives on the induction of cell differentiation, as assessed by the NBT reduction assay. The cells were incubated for 4 days with each derivative of vitamin D_3. 26,27-F_6-1,23(*S*),25-$(OH)_3D_3$ was nearly equipotent with 26,27-F_6-1,25-$(OH)_2D_3$ and was approximately 10-fold more active than 1,25-$(OH)_2D_3$. These data indicated that 23(*S*)-hydroxylation of 26,27-F_6-1,25-$(OH)_2D_3$ did not weaken its biological activity.

The hypercalcemic effects of 26,27-F_6-1α-OH-D_3 *in vitamin D-deficient rats.* 1α-OH-D_3 is easily metabolized to 1,25-$(OH)_2D_3$, an active form of vitamin D_3, via 25-hydroxylation in liver to show a hypercalcemic effect *in vivo* [21]. Therefore, 1α-OH-D_3 is one-half as potent as 1,25-$(OH)_2D_3$, although its binding affinity for 1,25-$(OH)_2D_3$ receptor is 100–1000 times lower than that of 1,25-$(OH)_2D_3$ [22]. Figure 4 shows the time course of the hypercalcemic effect after a single dose of 26,27-F_6-1α-OH-D_3. Even when provided at 500 pmol/100 g body weight, 26,27-F_6-1α-OH-D_3 failed to cause a significant increase in serum calcium levels in vitamin D-deficient rats (data not shown). 26,27-F_6-1α-OH-D_3 at 5000 pmol/100 g body weight increased serum calcium levels significantly to a degree similar to that of 500 pmol/100 g body weight of 1,25-$(OH)_2D_3$ 12 hr after the injection of each vitamin D_3 compound, with a maximal increase detected at 24 hr. A dose–response study on the basis of the serum calcium levels 24 hr after the injection of vitamin D_3 compounds, as shown in Table 1, demonstrated that biological potency of 26,27-F_6-1α-OH-D_3 was 10- to 100-fold lower than that of 26,27-F_6-1,25-$(OH)_2D_3$, which correlates well with their respective binding affinity for the 1,25-$(OH)_2D_3$ receptor [5]. These data suggested that 26,27-F_6-1,25-$(OH)_2D_3$ might show a hypercalcemic effect *in vivo* without being metabolized to 26,27-F_6-1,25-$(OH)_2D_3$.

Determination of serum levels of 26,27-F_6-1,25-$(OH)_2D_3$ *and* 1,25-$(OH)_2D_3$ *after injection of* 26,27-F_6-1α-OH-D_3 *and* 1α-OH-D_3. To confirm the hypothesis that 26,27-F_6-1α-OH-D_3 might not be hydroxylated efficiently at its C-25 position *in vivo*, serum levels of 26,27-F_6-1,25-$(OH)_2D_3$ were determined in vitamin D-deficient rats after injection of 26,27-F_6-1α-OH-D_3, in comparison with serum 1,25-$(OH)_2D_3$ levels after 1α-OH-D_3 injection. As shown in Table 2, 26,27-F_6-1,25-$(OH)_2D_3$ did not appear in serum 8 hr after injection of 26,27-F_6-1α-OH-D_3 (500 pmol/100 g body weight), which is in contrast to the significant appearance of serum 1,25-$(OH)_2D_3$ after injection of 1α-OH-D_3. Even after injection of 5000 pmol/100 g body weight of 26,27-F_6-1α-OH-D_3, serum levels of 26,27-F_6-1,25-$(OH)_2D_3$ were markedly lower than those of 1,25-$(OH)_2D_3$ after 500 pmol of 1α-OH-D_3. Taken together with the data showing that the metabolic clearance rate of 26,27-F_6-1,25-$(OH)_2D_3$ was no shorter than that of 1,25-$(OH)_2D_3$ (data not shown), it seemed likely that 26,27-F_6-1α-OH-D_3 may not undergo 25-hydroxylation as efficiently as 1α-OH-D_3.

DISCUSSION

In the past, many fluorinated analogs of vitamin D_3 were synthesized to clarify the mode of action of vitamin D_3. Fluorine mimics hydrogen in an atomic dimension and the strength of a carbon–fluorine bond exceeds that of a carbon–hydrogen bond, thus causing an increase in its stability [23, 24]. Therefore, it might be expected that, if fluorines are substituted for the hydrogens on C-26 and C-27 of 1,25-$(OH)_2D_3$, hydroxylations at these positions on the vitamin D molecule could not take place or at least might be inhibited markedly, thus minimizing the effect of modification at C-26 and C-27 positions [1, 5]. In this study, we demonstrated that exposure of HL-60 cells to 1,25-$(OH)_2D_3$ or 26,27-F_6-1,25-$(OH)_2D_3$

Table 1. Effects of various vitamin D_3 compounds on serum calcium levels in vitamin D-deficient rats

Vitamin D_3 compounds	Serum calcium levels (mg/dL)			
	Doses (pmol/100 g body wt)			
	0	50	500	5000
Control	4.8 ± 0.3			
$1,25\text{-}(OH)_2D_3$		5.5 ± 0.3*	6.4 ± 0.7†	
$F_6\text{-}1,25\text{-}(OH)_2D_3$		6.6 ± 0.6†		
$F_6\text{-}1\alpha\text{-OH-}D_3$			5.2 ± 0.3	7.6 ± 0.3†

Vitamin D-deficient rats were fed a low-calcium diet and given the indicated doses via a single intrajugular administration. At 24 hr after the injection, the rats were killed for the determination of serum calcium levels, in order to assess the potency of each vitamin D_3 analog to mobilize calcium from bone. Values are means ± SD of five rats per group.

* $P < 0.05$ (Student's t-test) vs control.

† $P < 0.01$ (Student's t-test) vs control.

Table 2. Serum levels of $26,27\text{-}F_6\text{-}1,25\text{-}(OH)_2D_3$ and $1,25\text{-}(OH)_2D_3$ after injection of $26,27\text{-}F_6\text{-}1\alpha\text{-OH-}D_3$ and $1\alpha\text{-OH-}D_3$ in vitamin D-deficient rats

Time (hr)	Serum $26,27\text{-}F_6\text{-}1,25\text{-}(OH)_2D_3$ (pmol/mL)		Serum $1,25\text{-}(OH)_2D_3$ (pmol/mL)
	$26,27\text{-}F_6\text{-}1\alpha\text{-OH-}D_3$ (500 pmol/100 g body wt)	$26,27\text{-}F_6\text{-}1\alpha\text{-OH-}D_3$ (5000 pmol/100 g body wt)	$1\alpha\text{-OH-}D_3$ (500 pmol/100 g body wt)
8	<0.05	0.27 ± 0.08	8.00 ± 2.13
12	0.08 ± 0.02	0.53 ± 0.32	2.72 ± 1.41

Serum levels of $26,27\text{-}F_6\text{-}1,25\text{-}(OH)_2D_3$ and $1,25\text{-}(OH)_2D_3$ were determined at the indicated times after injection of the indicated doses of $26,27\text{-}F_6\text{-}1\alpha\text{-OH-}D_3$ and $1\alpha\text{-OH-}D_3$, respectively, in vitamin D-deficient rats. After $26,27\text{-}F_6\text{-}1,25\text{-}(OH)_2D_3$ and $1,25\text{-}(OH)_2D_3$ were extracted and separated with HPLC as described under Materials and Methods, their levels were determined by a competitive protein binding assay using chick intestinal cytosol as a $1,25\text{-}(OH)_2D_3$ receptor source. Data are means ± SD of six vitamin D-deficient rats.

significantly induced the ability of the cells to hydroxylate the C-24 position of $1,25\text{-}(OH)_2[26,27\text{-}^3H]D_3$. However, even under the same conditions in which HL-60 cells acquire 24-hydroxylase activity, $26,27\text{-}F_6\text{-}1,25\text{-}(OH)_2D_3$ did not undergo 24-hydroxylation, thus suggesting that introduction of fluorine atoms at the C-26 and C-27 positions of $1,25\text{-}(OH)_2D_3$ may change chemical reactivity and/or conformation of the C-24 position of the vitamin D_3 molecule [22]. Furthermore, we have demonstrated that the hypercalcaemic effect of $26,27\text{-}F_6\text{-}1\alpha\text{-OH-}D_3$ in vitamin D-deficient rats was in good agreement with the expected one predicted from its binding affinity for $1,25\text{-}(OH)_2D_3$ receptor, compared with that of $26,27\text{-}F_6\text{-}1,25\text{-}(OH)_2D_3$ [5]. These phenomena were totally different from those of nonfluorinated counterparts in that the hypercalcemic effect of $1\alpha\text{-OH-}D_3$ was much higher than the expected potency on the basis of its binding affinity for the $1,25\text{-}(OH)_2D_3$ receptor. In fact, the appearance of 25-hydroxylated product of $26,27\text{-}F_6\text{-}1,25\text{-}(OH)_2D_3$ was delayed markedly, and its level was much lower than that of $1,25\text{-}(OH)_2D_3$ (Table 2). These data strongly suggest that $26,27\text{-}F_6\text{-}1\alpha\text{-OH-}D_3$ may not undergo 25-hydroxylation in the liver in contrast to $1\alpha\text{-OH-}D_3$, which is easily hydroxylated at its C-25 position in the liver to reveal a hypercalcemic effect [21]. In contrast to its 24- and 25-hydroxylation, $26,27\text{-}F_6\text{-}1,25\text{-}(OH)_2D_3$ easily underwent 23(S)-hydroxylation in both the *in vitro* HL-60 cell system (Fig. 1) and *in vivo* [18]. 23(S)-Hydroxylation of $1,25\text{-}(OH)_2D_3$ seemed to be an inactivation step because $1,23(S),25\text{-}(OH)_3D_3$ has neither intestinal calcium absorption nor bone calcium resorption activity [25]. In contrast to $1,23(S),25\text{-}(OH)_3D_3$, $26,27\text{-}F_6\text{-}1,23(S),25\text{-}(OH)_3D_3$ retained biological activity to the same degree as that of $26,27\text{-}F_6\text{-}1,25\text{-}(OH)_2D_3$ in the induction of HL-60 cell differentiation (Fig. 3) as well as in binding activity for the $1,25\text{-}(OH)_2D_3$ receptor [18]. These data suggested that configuration of the C-23 position might somehow be different between $26,27\text{-}F_6\text{-}1,25\text{-}(OH)_2D_3$ and $1,25\text{-}(OH)_2D_3$. The collective results of the present study suggest that introduction of fluorine atoms at C-26 and C-27 positions may inhibit hydroxylation at C-24 and C-25 positions and it also may cause conformational alterations in secondary or tertiary structure of the C-23 position of the side chain of the $1,25\text{-}(OH)_2D_3$ molecule.

In summary, substitution of fluorines for hydrogens

at C-26 and C-27 positions caused a significant change of chemical reactivities and/or conformation of the neighboring structure of the 1,25-$(OH)_2D_3$ side chain at least including the C-23, C-24 and C-25 positions.

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