Biological activity of 26,26,26,27,27,27-hexafluoro-1,25-dihydroxyvitamin D$_3$ in the chick

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Abstract. A newly synthesized fluorinated analogue of 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$), 26,26,26,27,27,27-hexafluoro-1,25-dihydroxyvitamin D$_3$ (26,27-F$_6$-1,25(OH)$_2$D$_3$) has been compared with 1,25(OH)$_2$D$_3$ as to its biological activity in vitamin D-deficient chicks. One day old, white Leghorn cockerels were fed a rachitogenic diet for 5 weeks. They were then given vehicle or 32.5, 130 or 325 pmol of 26,27-F$_6$-1,25(OH)$_2$D$_3$ or 1,25(OH)$_2$D$_3$ in a solution of propyleneglycol:ethanol (95:5 v/v) sc every day for 2 weeks. Twenty-four hours after the last dose, the animals were sacrificed and their femurs were removed. 26,27-F$_6$-1,25(OH)$_2$D$_3$ was more active than 1,25(OH)$_2$D$_3$ in stimulating growth, healing of rachitic cartilage visualized by soft X-ray radiography, elevation of serum inorganic phosphorus, and mineralization of rachitic bone. These biological differences between two compounds were observed only for the dose of 130 pmol. However, this fluorinated compound has less binding ability than 1,25(OH)$_2$D$_3$ to fetal chick intestinal cytosol receptors. The mechanism of the higher potency of this analogue is still unknown, but its affinity to the 1,25(OH)$_2$D$_3$ receptor does not account for the higher activity. Since 26-hydroxylation can be postulated as the inactivation step in vitamin D metabolism, these results suggest that the reason for increased activity of this fluorinated analogue is most likely its slower metabolism.

Since the discovery of 1α,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) as the most potent metabolite of vitamin D$_3$, there has been much interest in the preparation of structural analogues of this metabolite in order to obtain more active compounds and to enhance its original properties.

Various fluorine-substituted analogues of vitamin D have been synthesized and tested for biological activity. Among these compounds, 24,24-difluoro-1α,25-dihydroxyvitamin D$_3$ (24-F$_2$-1,25(OH)$_2$D$_3$) was the first analogue which had a higher activity than 1,25(OH)$_2$D$_3$ (1). The mechanism by which 24-F$_2$-1,25(OH)$_2$D$_3$ exerts a higher and longer activity than 1,25(OH)$_2$D$_3$ is still unclear. However, it is believed that the blocking of 24-hydroxylation plays an important role in this request.

Based on the postulation that 24- or 26-hydroxylation is the inactivation step in vitamin D metabolism, another fluorinated analogue of vitamin D$_3$, 26,26,26,27,27,27-hexafluoro-1α,25-dihydroxyvitamin D$_3$ (26,27-F$_6$-1,25(OH)$_2$D$_3$) has been synthesized (2). This reports shows that 26,27-F$_6$-1,25(OH)$_2$D$_3$ is more active than the natural hormone 1,25(OH)$_2$D$_3$ in the vitamin D-deficient chick system as described by Okamoto et al. (3).

Material and Methods

**Vitamin D compounds**

26,27-F$_6$-1,25(OH)$_2$D$_3$ was synthesized in our laboratory by the method of Kobayashi et al. (2). 1,25(OH)$_2$D$_3$ was purchased from Roussel UCLAF (Paris, France).
Animals

One-day-old, white Leghorn cockerels (purchased from the Otsubo Experimental Animal Farm, Nagasaki, Japan) were obtained and fed ad libitum a purified rachitogenic diet (1.3% calcium, 0.7 phosphorus) for 5 weeks. They were then given 26,27-F$_6$-1,25(OH)$_2$D$_3$ or 1,25(OH)$_2$D$_3$ or vehicle daily for 2 weeks.

Administration of compounds

Daily doses of 32.5, 130 or 325 pmol of 26,27-F$_6$-1,25(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_3$ were given in 0.1 ml solution of propylenglycol:ethanol (95:5, v/v) sc for two weeks. Chicks in the control group received only the vehicle. Chicks on the above-mentioned diet were divided into groups of five or six.

Twenty-four hours after the last dose was given, the animals were weighed and thereafter exsanguinated by cardiac puncture. The femurs were carefully removed and cleaned of adhering connective tissue. Length of each femur was measured from the head to the medial condyle with vernier calipers.

Determination of serum inorganic phosphorus and calcium

Twenty-four hours after the last dose was given, the blood was collected by cardiac puncture. Serum was harvested from the blood by centrifugation and used for the determination of inorganic phosphorus and calcium.

Inorganic phosphorus was determined by the colorimetric method of Chen et al. (4), whereas calcium was determined with 0.1% lanthanum chloride as diluent by means of a Hitachi atomic absorption spectrometer Model Z-8000 (5).

Measurement of bone ash

After the connective tissue was removed, femurs were extracted successively with 100% ethanol and 100% diethyl ether for 24 h, respectively, using a Soxhlet extractor. Fat-free bones were dried in a 160°C oven for 8 h until they reached constant weight and ashed in a muffle furnace at 650°C for 24 h.

Radiograms of the femurs were taken with a soft X-ray unit SOFRON SRO-M50 (Tanaka X-ray MFG, Co, Ltd, Tokyo, Japan) using 4.0 mA at 40 kV for 10 min. The focus-to-film distance was 100 cm, and the femurs were placed directly on the film pack. Films were developed for one minute in an X-ray film developer (Rendol, Fuji Photo Fil, Co, Ltd, Tokyo, Japan) at a temperature of 20°C.

The sensitivity of the X-ray method was to detect a 10% change in femur ash content.

Displacement of [26,27-3H]1,25(OH)$_2$D$_3$ from chick intestinal cytosol receptor by 1,25(OH)$_2$D$_3$ or 26,27-F$_6$-1,25(OH)$_2$D$_3$

Aliquots of either 1,25(OH)$_2$D$_3$ or 26,27-F$_6$-1,25-(OH)$_2$D$_3$ were dissolved in 95% ethanol. Triplicate determination

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<th>Table I</th>
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<td><strong>Comparison of the effect of 26,27-F$_6$-1,25(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_3$ on body weight, femur length, femur dry weight, percent femur ash, and serum calcium in chicks.</strong></td>
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<td>Treatment</td>
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<td>Control</td>
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<td>1,25(OH)$_2$D$_3$</td>
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<td>26,27-F$_6$-1,25(OH)$_2$D$_3$</td>
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Each group consisted of 5–6 chicks. The data are expressed as far from dry bone weight and percent of ash based on dry bone weight. The data are presented as means and 1 sd, *p* < 0.05, **p** < 0.01 vs control group. The fluorinated compound treated group vs 1,25(OH)$_2$D$_3$ treated group.
of displacement of [³H]-1,25(OH)₂D₃ from chick intestinal cytosol receptor (Yamasa Co., Ltd, Tokyo, Japan) by unlabelled compound was carried out as described by Shepard et al. (6).

Statistics
The results are expressed as mean ± 1 s.n. Student’s t-test was used to test the degree of significance.

Results
A significant increase in body weight was observed at the dose of 130 pmol daily of 26,27-F₆-1,25(OH)₂D₃, but not at the same dose of 1,25(OH)₂D₃. However, at the dose of 325 pmol daily, both compounds showed a significant and similar increase (Table 1).

Femur length was significantly increased at the daily dose of 130 pmol of 26,27-F₆-1,25(OH)₂D₃, but not at the same dose of 1,25(OH)₂D₃. At the dose of 325 pmoles daily, both compounds showed a significant increase (Table 1).

Both compounds caused an increase in serum calcium levels in a dose-related fashion. Serum calcium levels were significantly higher at a daily dose of 130 pmol of 26,27-F₆-1,25(OH)₂D₃ than of 1,25(OH)₂D₃ (Table 1).

Control chicks had a severe hypophosphatemia and low bone ash content. As shown in Fig. 1, both compounds increased serum inorganic phosphorus levels, but the fluoro-analogue was clearly

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**Fig. 1.**
Dose-response of serum inorganic phosphorus (P) levels to daily dose of 26,27-F₆-1,25(OH)₂D₃ (Δ—Δ) or 1,25(OH)₂D₃ (Ο—Ο). The data are presented as means and 1 s.n. *p < 0.01; ^p < 0.05 vs control group, +; p < 0.05 vs 1,25(OH)₂D₃ treated group.

**Fig. 2.**
Soft X-ray radiography of femurs. Control group (left side, upper and lower); 1,25(OH)₂D₃ treated groups (upper); 26,27-F₆-1,25(OH)₂D₃ treated groups (lower).

**Fig. 3.**
Competitive protein binding assay of 1,25(OH)₂D₃ and 26,27-F₆-1,25(OH)₂D₃. Percent specific binding of 1,25(OH)₂D₃[26,27³H]D₃ is plotted against log concentration. 1,25(OH)₂D₃ (Δ—Δ); 26,27-F₆-1,25(OH)₂D₃ (Ο—Ο). Values are expressed as the mean of duplicate determinations.
more active than 1,25(OH)2D3. Likewise, mineralization of rachitic bone, as assessed by percent femur ash, was more effectively stimulated by 26,27-F6-1,25(OH)2D3 than by 1,25(OH)2D3 (Table 1). At the daily dose of 325 pmol, rat femurs treated with the fluorinated analogue revealed a decrease in ash content compared with that at the daily dose of 130 pmol.

The soft X-ray radiographic appearance of the femurs are shown in Fig. 2. In vitamin D-deficient chicks, the metaphyses, condyles, and the femoral heads are dim and irregular, reflecting lack of mineralization. Also, bone density is low and trabecular patterns are obscure. At the dose of 130 pmol/day, healing changes were apparent with both compounds. The border of the femoral head, the trochanter, and both condyles became sharper in appearance in a dose-dependent fashion. 26,27-F6-1,25(OH)2D3 was more potent than 1,25(OH)2D3 in healing rachitic bone. The bone density at the dose of 325 pmol/day was clearly increased as compared with that of 130 pmol in 1,25(OH)2D3 treated chick femur, whereas it was clearly decreased in 26,27-F6-1,25(OH)2D3 treated chick femur. The binding abilities of 26,27-F6-1,25(OH)2D3 and 1,25(OH)2D3 for fetal chick intestinal cytosol receptor are shown in Fig. 3. Binding affinity of 26,27-F6-1,25(OH)2D3 was found to be less than that of 1,25(OH)2D3. The ratio of 50% displacing dose was 0.6.

Discussion

Our studies clearly show that 26,27-F6-1,25(OH)2D3 is more bioactive than 1,25(OH)2D3 in chicks. However, 26,27-F6-1,25(OH)2D3 has less binding ability than 1,25(OH)2D3 to fetal chick intestinal cytosol receptors.

The reason for this increased activity of 26,27-F6-1,25(OH)2D3 is unknown, but it is clear that the enhanced activity of this compound results from the fluorine-substitution of the 26- and 27- position.

In general, it is well known that 1- and 25-hydroxylations are required for vitamin D function. Since substitution of 1- and 25-hydroxylations with fluorine markedly reduces the activity of vitamin D compounds (7, 8), substituted fluorine is thought to block hydroxylation of these positions. Among other fluorinated compounds, the 24-fluoro group and the 25-fluoro group do not act as hydroxyl groups in their binding to chick intestinal cytosol receptors (9, 10) and also in their ability to heal rickets in rats (11). Based on these findings, three possible explanations can be given for the higher bioactivity of 26,27-F6-1,25(OH)2D3. First, 26,27-F6-1,25(OH)2D3 is stronger than 1,25(OH)2D3 in its binding activity to the 1,25(OH)2D3 receptors; second, that the fluorinated compound is degraded more slowly than 1,25(OH)2D3, and third, that the compound enters the target cells more easily than 1,25(OH)2D3.

The first explanation can be excluded since the binding activity of 26,27-F6-1,25(OH)2D3 to the chick intestinal cytosol receptor was found to be less than that of 1,25(OH)2D3. With regard to the third possibility, the fluorinated compound may have greater membrane permeability than 1,25(OH)2D3, but the hydrophobic fragment values of 26,27-F6-1,25(OH)2D3 and 1,25(OH)2D3 are 7.335 and 7.237, respectively. This small difference may not be sufficient to account for the higher bioactivity of the fluorinated compound as compared with 1,25(OH)2D3.

Although the metabolism and degradation of this fluorinated compound are not clearly understood, fluorine-substitution at the 26- and 27- positions is thought to block hydroxylation at these positions. Since 26-hydroxylation can be postulated as the inactivation step in vitamin D metabolism, blocking of 26- and 27- hydroxylation of 26,27-F6-1,25(OH)2D3 may lead to a decrease in plasma clearance rate or intracellular degradation rate. Nagata et al. (12) demonstrated that accumulation of a highly active metabolite, 26,27-F6-1,23,25(OH)3D3, was shown in the intestine after administration of [3H]26,27-F6-1,25(OH)2D3 to vitamin D-deficient rats, and that the serum clearances of 1,25(OH)2D3 and F6-analogue were similar. Taken together, it may be postulated that a possible decrease in intracellular degradation of 26,27-F6-1,25(OH)2D3 leads to an increase in its biological action and to accumulation of a highly active metabolite in the intestine. At the daily dose of 325 pmol of 26,27-F6-1,25(OH)2D3, chick femurs revealed a decrease in ash content and bone density compared with those at the daily dose of 130 pmol. This may be explained as signs of toxicity.

Similar enhancement of the biological activity of other fluorinated compounds has been reported for 24-F2-1,25(OH)2D3 (1, 13), one of the analogues of 1,25(OH)2D3, as well as for steroid hormone. The
results on the biological activity of 26,27-F$_6$-1,25(OH)$_2$D$_3$ are in accordance with previous findings in rachitic rats (14). Since this newly synthesized biologically active analogue of vitamin D$_3$, 26,27-F$_6$-1,25(OH)$_2$D$_3$, is more active than the natural hormone, it can be of considerable use not only for clinical application but also for unravelling the mechanisms of vitamin D action and metabolism.

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References


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