A Highly Potent 26,27-Hexafluoro-1α,25-dihydroxyvitamin D₃ on Calcification in SV40-Transformed Human Fetal Osteoblastic Cells


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Abstract. 26,27-hexafluoro-1α,25-dihydroxyvitamin D₃ (F₆-D₃) has been reported to be 5–10 times more potent than 1α,25-dihydroxyvitamin D₃[1,25(OH)₂-D₃] in biological systems in vivo and in vitro. However, the effect of F₆-D₃ on bone formation has yet to be clarified. In the present study, we investigated the effect of F₆-D₃ on SV40-transfected human fetal osteoblastic cells (SV-HFO) and found it to be about 100 times more potent than that of 1,25(OH)₂D₃ in stimulating calcification. F₆-D₃ was also about 100 times more effective than 1,25(OH)₂D₃ in enhancing the expression of mRNA for alkaline phosphatase (ALP), osteocalcin (OCN), and osteopontin (OPN). In the presence of 10⁻³ M F₆-D₃ and 10⁻⁶ M 1,25(OH)₂D₃, the calcification began on day 9 and increased up to day 19. Expression of mRNA for ALP and OCN reached a maximum on day 4 and thereafter declined. On the other hand, when osteoblastic cells were incubated with a low level of [1β⁻3H]-F₆-D₃ or [1β⁻3H]-1,25(OH)₂D₃, each radioactive peak could not be detected. However, on the incubation of osteoblastic cells and radioactive substrate in the presence of ketoconazole, a selective inhibitor of CYP24, a clear peak for each substrate was detected. This suggested that F₆-D₃ as well as 1,25(OH)₂D₃ is metabolized by CYP24. Osteoblastic cells were incubated with 10⁻⁸ M [1β⁻3H]-F₆-D₃ or 10⁻⁶ M [1β⁻3H]-1,25(OH)₂D₃ for 4, 9, and 14 days. A small peak of 1,25(OH)₂D₃ was observed and thereafter its level decreased. In addition, two unknown peaks increased when the culture period was extended. In the case of F₆-D₃, peaks of F₆-D₃ and 26,27-hexafluoro-23-oxo-1α,25(OH)₂D₃(23-oxo-F₆) were clearly detected, the latter being about 4 times higher than the former. Both peaks were retained up to day 14. The amount of unlabeled F₆-D₃ and 23-oxo-F₆ calculated from the specific radioactivity in the cells may be similar to the amount of 1,25(OH)₂D₃ and its metabolites. The strong activity of F₆-D₃ in stimulating calcification may be due to the fact that F₆-D₃ is much more potent than 1,25(OH)₂D₃ in enhancing the expression of mRNA for ALP, OCN, and OPN and that the amount of F₆-D₃ and 23-oxo-F₆ accumulated in the cells is much greater than that of 1,25(OH)₂D₃ and its metabolite.

Key words: 26,27-hexafluoro-1α,25-dihydroxyvitamin D₃ — 26,27-hexafluoro-23-oxo-1α,25-dihydroxyvitamin D₃ — 1α,25-dihydroxyvitamin D₃ — Calcification — SV40-transformed human fetal osteoblastic cells

26,27-hexafluoro-1α,25-dihydroxyvitamin D₃(F₆-D₃) is a hexafluorinated analogue of the active form of vitamin D₃, 1α,25-dihydroxyvitamin D₃[1,25(OH)₂D₃], synthesized by Kobayashi et al. [1]. F₆-D₃ was 5–10 times more potent than 1,25(OH)₂D₃ in increasing the levels of serum Ca and P, in stimulating intestinal Ca absorption, and in enhancing bone Ca mobilization [2]. However, in a 48-hr culture system using fetal rat limb bones, the effect of F₆-D₃ on bone resorption was found to be similar to that of 1,25(OH)₂D₃ [3]. When we used neonatal mouse parietal bones and extended the culture period from 48 to 144 hr, F₆-D₃ was found to be about 10 times more potent than 1,25(OH)₂D₃ in stimulating bone resorption [4].

Although F₆-D₃ was more potent than 1,25(OH)₂D₃ in biological systems in vivo and in vitro [5], its effect on bone formation has yet to be clarified. In the present study, we investigated the effect of F₆-D₃ on calcification using human osteoblastic cells (SV-HFO; SV40-transformed human fetal osteoblastic cells) [6]. As F₆-D₃ was found to be about 100 times more potent than 1,25(OH)₂D₃ in stimulating calcification, we further examined the expression of mRNA for alkaline phosphatase (ALP), osteocalcin (OCN) and osteopontin (OPN), and metabolism of the two compounds. As shown in Figure 1, F₆-D₃ is converted to 26,27-hexafluoro-1α,23(S),25-trihydroxy D₃(23-F₆-D₃) which is further converted to 26,27-hexafluoro-23-oxo-dihydroxyvitamin D₃(23-oxo-F₆).

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Fig. 1. Metabolism of 26,27-hexafluoro-1,25-dihydroxyvitamin D₃.

Materials and Methods

Materials

1,25(OH)₂D₃ was purchased from Wako Pure Industries, Ltd. F₆-D₃, 23-F₆-D₃, 23-oxo-F₆, [1β⁻³H]-1,25(OH)₂D₃ (772 kBq/nmol), and [1β⁻³H]-F₆-D₃ (32 kBq/nmol) were from Sumitomo Pharmaceutical Co. Ltd. 1x, 24,25(OH)₂D₃ was a gift from Dr. T. Okano. Fetal calf serum (FCS) was purchased from Irvine Scientific Co. Ltd.

Cell Culture

Human fetal osteoblastic (HFO) cells were obtained at autopsy from fetuses aborted in the 26th week of gestation and were treated at P4 with wild-type SV40. Osteoblastic cells were obtained in 2 ml of α MEM (ICN Biomedical Inc.) containing 10% FCS in 6-well plates at 1.2 × 10⁶ cells/well. After 3 days culture, the medium was changed to α MEM containing 10% FCS, 5 mM HEPES, 10 mM β-glycerophosphate, and 50 μg/ml of ascorbic acid. The medium was then replaced every 2–3 days with new medium containing tritium-labeled compound.

von Kossa Staining and Ca Assay

On removal of the medium, 2 ml of 10% formalin was added and fixed for 10 min. After the removal of formalin, the cells were washed with water 2–3 times. One milliliter of 5% AgNO₃ was added and the cells were irradiated with ultraviolet light for 5 min. Ag⁺ binds to phosphate ion and Ca²⁺ was released from the cells. Calcified areas are dyed black. AgNO₃ solution was diluted with 5% HNO₃ and used to assay Ca with an atomic absorption spectrophotometer (Hitachi Co. Japan).

Northern Blot Analysis

Total cellular RNA was extracted from osteoblastic cells using the acid guanidium/phenol/chloroform method [7]. For Northern blotting, 30 μg of total RNA was resolved by electrophoresis in a 1.5% agarose-formaldehyde gel and transferred onto nylon membrane (Hybond N, Amersham Corp.) and then hybridized with ³²P-labeled cDNA probe as reported [7]. The signal were quantified densitometrically using a Bio-Image analyzer (BAS-2000, Fuji Film, Tokyo, Japan). Human OCN cDNA was a gift from Dr. S. Nomura. Human ALP cDNA (information for ATCC number 59623) was purchased from American Type Culture Collection Corp.. Human OPN cDNA was amplified by RT-PCR according to the method of Young [8]. The primers used were 5’-CTTTTCAAGTGAGCGGAGTTTGGATCAGCCGTA-3’ (sense) and 5’-ACAGGGATTTCCATGAAGCC-3’ (antisense).

Assay of Hydroxyproline

A hydroxyproline assay was performed by the method of Nishino et al. [9]. A screw test tube containing 0.4 ml of cell suspension and 0.4 ml of 35% HCl was heated at 115°C for 15 hr. After removal of the cap, the test tube was put in a desicator with particle NaOH and sucked under low pressure. After drying, 1.5 ml of isopropanol and 0.5 ml of chloramine T were added to the test tube. After 4 min, 1 ml of Ehrlich reagent was added and the tube was left at 25°C for 18 hr. The absorbance was then measured at 562 nm.

Extraction of Metabolites from the Cells

The medium and cells were added to the tube and centrifuged at 2200 rpm for 5 min at 4°C [10]. Then the supernatant was removed, 2 ml Ca²⁺-, Mg²⁺-free phosphate buffer was added, and the cells were sonicated for 2 min with Astrason XL. The homogenate was extracted first with a mixture of 5 ml of ethyl acetate and 1 ml of tetrahydrofuran and then with 4 ml of ethyl acetate twice. The extracted ethyl acetate was dried with an evaporator. The dried material was dissolved in 96% n-hexane:4% isopropanol and passed through a Sep-Pak cartridge (Waters Associates, Milford, MA, USA). F₆-D₃ or 1,25(OH)₂D₃ and each metabolite were eluted with 80% n-hexane:20% isopropanol. The eluent was dripped with an evaporator and subjected to HPLC with a straight-phase Zorbax-5IL Column (4.6 × 250 mm, Dupont Instruments, Wilmington, DE) at a flow rate of 1.5 ml/min., [1β⁻³H]-F₆-D₃ and its metabolites were eluted with 90% n-hexane:10% isopropanol, [1β⁻³H]-1,25-(OH)₂D₃ and its metabolites were eluted with 96% dichloromethane:4% methanol. The eluent was collected in 0.5 min fractions and then the radioactivity was measured with a liquid scintillation counter. The recovery of radioactivity in HPLC system was 80% and above.