Isolation, Identification and Biological Activity of 24R,25-Dihydroxy-3-epi-vitamin D₃: A Novel Metabolite of 24R,25-Dihydroxyvitamin D₃ Produced in Rat Osteosarcoma Cells (UMR 106)

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Summary We recently identified 1 α ,25-dihydroxy-3-epi-vitamin D₃ [1 α ,25(OH)₂-3-epi-D₃] as a metabolite of 1 α ,25-dihydroxyvitamin D₃ [1 α ,25(OH)₂D₃] produced in rat osteosarcoma cells (UMR 106). We now report the isolation of 24R,25-dihydroxy-3-epi-vitamin D₃ [24R,25(OH)₂-3-epi-D₃] as a metabolite of 24R,25-dihydroxyvitamin D₃ [24R,25(OH)₂D₃] by high-performance liquid chromatography (HPLC) with chiral column and its structure assignment by proton nuclear magnetic resonance (¹H-NMR) and liquid chromatography-mass spectrometry (LC-MS) analysis. We also demonstrated the production of 24R,25(OH)₂-3-epi-D₃ in two other cell lines [human colon carcinoma cells (Caco-2) and porcine kidney cells (LLC-PK₁)] which were previously shown to convert 1 α ,25(OH)₂D₃ into 1 α ,25(OH)₂-3-epi-D₃. It can be seen that the production of 24R,25(OH)₂-3-epi-D₃ from 24R,25(OH)₂D₃ is lower than that of 1 α ,25(OH)₂-3-epi-D₃ from 1 α ,25(OH)₂D₃ in all the cells studied. 24R,25(OH)₂-3-epi-D₃ was found to be inactive in terms of its ability to bind to the vitamin D receptor (VDR), in inhibiting proliferation and in inducing differentiation of human promyelocytic leukemia cells (HL-60). Thus, our study indicates that the C-3 epimerization pathway is common to both 1 α ,25(OH)₂D₃ and 24R,25(OH)₂D₃ and may play an important role in modulating the concentration and the biological activity of these two major vitamin D₃ metabolites in target tissues.

Key Words 24R,25(OH)₂D₃, 24R,25(OH)₂-3-epi-D₃, 1 α ,25(OH)₂D₃, 1 α ,25(OH)₂-3-epi-D₃, metabolism

Vitamin D₃ is metabolized into 25-hydroxyvitamin D₃ [25(OH)D₃] in the liver and subsequently into 1 α ,25-dihydroxyvitamin D₃ [1 α ,25(OH)₂D₃] or 24R,25-dihydroxyvitamin D₃ [24R,25(OH)₂D₃] in the kidney (1, 2). 1 α ,25(OH)₂D₃ is known to be the most active form of vitamin D₃ and generates its biological actions through genomic as well as non-genomic pathways (3). 24R,25(OH)₂D₃ is considered to be an inactive form of vitamin D₃ although there are several reports demonstrating its potentials in increasing bone volume and strength at pharmacological doses (4, 5). 1 α ,25(OH)₂D₃ is further metabolized in its target tissues through modifications of both the side chain (C-24 and C-23 oxidation pathways) and the A-ring (C-3 epimerization pathway). The C-24 oxidation pathway (the major side chain metabolic pathway), initiated by

C-24 hydroxylation, leads to the conversion of 1 α ,25(OH)₂D₃ into the final product, calcitroic acid (6–9). The C-23 oxidation pathway (the minor side chain metabolic pathway), initiated by C-23 hydroxylation, leads to the conversion of 1 α ,25(OH)₂D₃ into the final product, 1 α ,25(OH)₂D₃-26,23-lactone (10–13).

Recently, a novel A-ring modification metabolic pathway of 1 α ,25(OH)₂D₃ was identified in human keratinocytes, which is initiated by epimerization of the hydroxyl group at C-3, the C-3 epimerization pathway (14). The C-3 epimerization results from a change in the orientation of the hydroxyl group at C-3 position from the natural (β) to epi (α) producing a novel metabolite, 1 α ,25-dihydroxy-3-epi-vitamin D₃ [1 α ,25(OH)₂-3-epi-D₃]. Further studies indicated that the C-3 epimerization pathway is target cell specific (15–18). 1 α ,25(OH)₂-3-epi-D₃ was also identified as a circulating metabolite of 1 α ,25(OH)₂D₃ in rats treated with pharmacological doses of 1 α ,25(OH)₂D₃, indicat-

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ing that the C-3 epimerization pathway is also functional *in vivo* (19). The binding of 1 α ,25(OH)₂-3-epi-D₃ to vitamin D receptor (VDR) when compared to that of 1 α ,25(OH)₂D₃ has been shown to be significantly reduced (18, 20). As a result, 1 α ,25(OH)₂-3-epi-D₃, when compared to 1 α ,25(OH)₂D₃, was found to be less active in stimulating intestinal calcium absorption and mobilizing calcium from bone (20). These findings suggest that like the well-established C-24 oxidation pathway, the newly discovered C-3 epimerization pathway appears to play an important role in the inactivation of the hormone. We observed that the C-3 epimerization pathway is expressed in some cell lines [e.g.: rat osteosarcoma cells (ROS 17/2.8)] in which the C-24 oxidation pathway is not expressed (16). This finding led us to hypothesize that the C-3 epimerization pathway is an alternate metabolic pathway of 1 α ,25(OH)₂D₃ in some tissues which lack the C-24 oxidation pathway.

In a recent study, Higashi et al. (21) reported for the first time the identification of the 24R,25(OH)₂-3-epi-D₃-24-glucuronide from the bile of rats administered pharmacological doses of 24R,25(OH)₂D₃. Higashi et al. (22) also identified 24R,25(OH)₂-3-epi-D₃ in the blood of rats dosed with 24R,25(OH)₂D₃ using high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS). However, by LC-MS and GC-MS technique, it is not possible to assign stereochemically the configuration of C-3 hydroxyl group. In the present study we first examined the metabolism of 24R,25(OH)₂D₃ in rat osteosarcoma cells (UMR 106), which are known to convert 1 α ,25(OH)₂D₃ into 1 α ,25(OH)₂-3-epi-D₃ (16, 18) and then identified 24R,25(OH)₂-3-epi-D₃ using proton nuclear magnetic resonance (¹H-NMR). In addition, the production of 24R,25(OH)₂-3-epi-D₃ in two other cell lines [human colon carcinoma cells (Caco-2) and porcine kidney cells (LLC-PK₁)] and biological activities of 3-epimer are described in this paper.

MATERIALS AND METHODS

Vitamin D₃ compounds. Authentic compounds of 24R,25(OH)₂-3-epi-D₃ and 1 α ,25(OH)₂-3-epi-D₃ were synthesized by Hatakeyama et al. of Nagasaki University, Japan. Synthetic compounds of 24R,25(OH)₂D₃ and 1 α ,25(OH)₂D₃ were obtained from Solvay-Duphar Co. (Weesp, The Netherlands). [26,27-methyl-³H]-1 α ,25(OH)₂D₃ (179 Ci/mmol) and [23,24(n)-³H]-25(OH)D₃ (82 Ci/mmol) were purchased from Amersham Pharmacia Biotech Ltd. (Buckinghamshire, UK). All the vitamin D₃ compounds were dissolved in ethanol and quantified by ultra violet (UV) spectroscopic analysis assuming an extinction coefficient (λ_{max} 265 nm) of 18,200 dm³/mol/cm.

Cells and cell culture. UMR 106 cells, Caco-2 cells and LLC-PK₁ cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Human promyelocytic leukemia cells (HL-60) were kindly provided by Dr. M. Inaba, Osaka City University Medical School. UMR 106 cells were maintained in

Dulbecco's modification Eagle's medium supplemented with 10% fetal calf serum (FCS) and antibiotics [penicillin (100 IU/mL) and streptomycin (100 μ g/mL)]. Caco-2 cells were maintained in Minimum essential medium (Eagle's medium) supplemented with 10% FCS, antibiotics and 1% non-essential amino acids. LLC-PK₁ cells were maintained in Medium 199 supplemented with 5% FCS and antibiotics. Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air in which the medium was changed every 3 d. For the metabolism studies, UMR 106 cells, Caco-2 cells and LLC-PK₁ cells, 2 $\times$ 10⁶ cells were seeded in 150-mm plates and grown to confluence. For the cell proliferation and cell differentiation assays, HL-60 cells were maintained in continuous culture in RPMI 1640 medium supplemented with 10% dextran-coated charcoal-treated FCS and kanamycin (0.06 mg/mL) at 37°C in a humidified atmosphere of 5% CO₂ in air. The doubling time of HL-60 cells was approximately 24 h. For synchronization at S phase, cells (4 $\times$ 10⁵) were cultured in 30 mL of RPMI 1640 medium for 24 h and subsequently cultured for 16 h in RPMI 1640 medium supplemented with 2.5 mM thymidine. After washing with calcium, magnesium-free phosphate buffered saline [PBS (-)] twice, the synchronization of cell cycle was repeated in the same manner, and the cells thus obtained were used for the cell proliferation and cell differentiation assays.

High-performance liquid chromatography (HPLC). Straight-phase HPLC analysis of the cell culture lipid extracts was performed with a model 600 pump and a model 996 photodiode array detector (Waters Associates, Milford, MA, USA) to monitor UV absorbing material at 265 nm. A Zorbax-SIL column (4.6 $\times$ 250 mm, Dupont Instruments, Wilmington, DE, USA) was eluted with a solvent mixture of hexane/2-propanol/methanol (88/10/2, vol/vol/vol) at a flow rate of 1.0 mL/min (HPLC system-I). A Sumichiral OA-2000 column (4.6 $\times$ 250 mm, Sumika Chemical Analysis Service, Ltd., Osaka, Japan) was eluted with a solvent mixture of 2-propanol/hexane (94.5/5.5, vol/vol) at a flow rate of 1.0 mL/min (HPLC system-II).

¹H-nuclear magnetic resonance (NMR) analysis. The 500-MHz ¹H-NMR spectra of the vitamin D₃ compounds and the isolated metabolite were measured on a Varian VXR-500 (¹H: 499.9 MHz). Synthetic compounds (50 μ g each) and the isolated metabolite (2 μ g) were dissolved in 40 μ L of deuterized chloroform (CDCl₃) containing a very small portion of CHCl₃ (7.24 ppm, used as an internal standard for ¹H-NMR spectroscopy) and transferred into a nano probe. To obtain 2D spectra, 128 experiments resulting in 1 K data points for a spectral width of 3,200 Hz were measured, and the time of domain data were multiplied with a squared sine-bell function for 2D COSY and a shifted Gaussian function for 2D NOESY, respectively. The 90 pulse width was 11 μ s and an MLEV-17 (Malcom Levitt) mixing sequence of 600 ms was used for 2D NOESY.

Liquid chromatography-mass spectrometry (LC-MS) analysis. LC-MS was carried out on a TSQ 700 triple-

stage quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with an atmospheric pressure chemical ionization (APCI) source in the positive ion mode. The HPLC system consisting of a pump (Model 600 MS, Waters Associates, Milford, MA, USA), an auto-injector (Model 717, Waters Associates, Milford, MA, USA) and a TSK-gel ODS-80Ts column (4.6×150 mm, $5 \mu\text{m}$; Tosoh Co., Ltd., Tokyo, Japan) was used. As a mobile phase, a solvent mixture of 0.1% acetic acid/acetonitrile (2/8, vol/vol) was used and HPLC was run at the flow rate of 1.2 mL/min. Vitamin D₃ compounds and the isolated metabolite were applied to LC-MS and mass spectra were obtained by averaging each peak and subtracting the background.

Metabolism of 24R,25(OH)₂D₃ and 1 α ,25(OH)₂D₃. Multiple experiments using confluent UMR 106 cells, Caco-2 cells and LLC-PK₁ cells were performed. The cells were incubated with $10 \mu\text{M}$ of either 24R,25(OH)₂D₃ or 1 α ,25(OH)₂D₃ in 10 mL media containing 1% bovine serum albumin (BSA) and antibiotics at 37°C. After 48 h, the incubations were terminated with 10 mL of methanol and the lipids from both cells and media were extracted for HPLC analysis using the procedure described previously (7). Control incubations containing only media and substrate without cells were performed to ensure that no metabolic conversion occurred in the absence of cells.

Vitamin D receptor (VDR) binding assay. VDR binding affinity of the vitamin D₃ compounds were tested using a calf-thymus VDR assay kit (Yamasa Co., Chiba, Japan). The receptor preparation was incubated at 20°C for 1 h with increasing concentrations of the compounds and then [26,27-methyl-³H]-1 α ,25(OH)₂D₃ (15,000 dpm) was added and incubated for 1 h at 20°C and VDR binding affinity was calculated as described previously (23).

Vitamin D binding protein (DBP) binding assay. DBP binding affinity of the vitamin D₃ compounds were tested using vitamin D-deficient rat plasma (24). The displacement of [23,24(n)-³H]-25(OH)D₃ from 1:70,000 diluted vitamin D-deficient rat plasma in 3.5 mM barbiturate buffer (pH 8.6) containing 0.13 M NaCl and 0.1% ovalbumin was measured following the addition of the compounds as described previously (23).

Flow cytometry. HL-60 cells (10^5 cells/well) were placed in 24-well tissue culture plates and cultured for 3 d with vitamin D₃ compounds (10^{-9} – 10^{-6} M). To reduce the effects of contact inhibition, control cells were adjusted to 60–70% confluency at the time of FACS analysis. Each group of cells was collected and washed with PBS (–). Then, the cells were resuspended in PBS (–) containing 0.2% Triton-X and 100 μg RNase, and incubated at 37°C for 1 h. Cells were washed with PBS (–) and incubated with 0.5 mL of DNA-staining solution containing propidium iodide (50 μg /mL) at 4°C for 20 min. The cells were analyzed with a flow cytometer equipped with an argon laser (488 nm, Becton Dickinson FACScanTM) and cell cycle distribution was analyzed by ModifiT LT (Verity).

Cell surface CD11b antigen expression analysis. HL-

60 cells (10^5 cells/well) were placed in 24-well tissue culture plates, and cultured for 3 d in RPMI-1640 medium with vitamin D₃ compounds or the isolated metabolite (10^{-9} – 10^{-6} M) under the same conditions as those of flow cytometry. Each group of cells was then collected and washed with PBS (–) once. Then, the cells (2×10^5 cells) were resuspended in 100 μL diluent solution containing 1% BSA and 1% sodium azide and incubated with 10 μL human monoclonal FITC conjugated CD11b antibody (Sigma, St. Louis, USA) for 30 min at room temperature. The cells were washed once with diluent solution and then fixed in 300 μL of PBS (–) containing 2% paraformaldehyde. Fluorescence was detected on a Becton Dickinson FACScanTM at excitation wavelength of 490 nm and emission wavelength of 520 nm. Results were recorded as the mean fluorescence index, which is the product of the % fluorescence and the mean fluorescence intensity, with 10^4 cells being counted per treatment.

Statistical analysis. The results are expressed as mean $\pm$ SE. Significance levels were determined by Student-*t*-test; a value of $p < 0.05$ was considered statistically significant.

RESULTS

Metabolism of 24R,25(OH)₂D₃ in UMR 106 cells

Lipid extracts from the media and cells incubated with $10 \mu\text{M}$ of 24R,25(OH)₂D₃ for 48 h were applied to HPLC system-I (Fig. 1A). The major peak corresponding to authentic standards of 24R,25(OH)₂D₃ (8.04 min) and 24R,25(OH)₂-3-epi-D₃ (8.03 min) was collected in a single fraction eluting between 7 and 10 min. The eluate was then subjected to HPLC system-II for the separation of 24R,25(OH)₂-3-epi-D₃ (19.23 min) from 24R,25(OH)₂D₃ (21.06 min) (Fig. 1B). The novel less polar peak (metabolite M), which possesses the characteristic UV chromophore of vitamin D₃ (λ_{min} 228 nm, λ_{max} 265 nm) (Fig 1B, inset) exhibited an identical retention time with that of authentic standard 24R,25(OH)₂-3-epi-D₃. The metabolite corresponding to peak M was not formed when 24R,25(OH)₂D₃ was incubated with the medium alone (control). It is clear from the HPLC chromatograms shown in Fig. 1B that metabolite M is the major metabolite of 24R,25(OH)₂D₃ produced by UMR 106 cells. Metabolite M was collected in sufficient quantity to permit identification by ¹H-NMR and LC-MS analysis. The minor unknown peak (metabolite U), which possesses the characteristic UV chromophore of previtamin D₃ (λ_{min} 228 nm, λ_{max} 260 nm), was not identified in the present study due to the unavailability of sufficient amounts of the compound. The other metabolites were not produced in UMR 106.

Identification of metabolite M by ¹H-NMR and LC-MS analysis

The proton chemical shifts assigned by 2D COSY and 2D NOESY spectra of authentic standards of 24R,25(OH)₂D₃, 24R,25(OH)₂-3-epi-D₃ and the metabolite M are summarized in Table 1. In the ¹H-NMR spectrum, the most pronounced difference between

Fig. 1. Metabolism of 24R,25(OH)₂D₃ in cultured UMR 106 cells.

A, upper panel: The HPLC profile (HPLC system-I) and UV spectra of the lipid extracts from UMR 106 cells incubated with 10 μ M of 24R,25(OH)₂D₃ for 48 h. 25(OH)D₃ is used as an internal standard (I.S.). The peak 3 exhibits an identical retention time (8.03 min) with that of 24R,25(OH)₂D₃ and 24R,25(OH)₂-3-epi-D₃. The HPLC fraction between 7 min and 10 min from HPLC system-I was collected for further purification. A, lower panel: The HPLC profiles (HPLC system-I) of authentic standards of 24R,25(OH)₂D₃ and 24R,25(OH)₂-3-epi-D₃. B, upper panel: The HPLC profile (HPLC system-II) and UV spectra of the fraction obtained from the HPLC system-I. Peaks M and S exhibit identical retention times (19.23 min and 21.06 min, respectively) with those of 24R,25(OH)₂-3-epi-D₃ and 24R,25(OH)₂D₃. The identity of peak U is not established. B, lower panel: The HPLC profiles (HPLC system-II) of authentic standards of 24R,25(OH)₂D₃ and 24R,25(OH)₂-3-epi-D₃.

24R,25(OH)₂D₃ and 24R,25(OH)₂-3-epi-D₃ was the chemical shift of H-3 [24R,25(OH)₂D₃: 3.93 ppm, 24R,25(OH)₂-3-epi-D₃: 3.86 ppm]. The chemical shift of H-3 of the metabolite M was also observed at the same position of 24R,25(OH)₂-3-epi-D₃ in upfield when compared to 24R,25(OH)₂D₃. Such an upfield shift appears to be responsible for the C-3 epimerization of 24R,25(OH)₂D₃. The resonances from the protons other than H-3 were proof of unchanged structure of the side-chain as well as the cis-triene system.

Figure 2 shows the LC-MS spectra of 24R,25(OH)₂-3-

epi-D₃ (R.T., 4.31 min), 24R,25(OH)₂D₃ (R.T., 4.31 min) and the metabolite M (R.T., 4.31 min). As expected, 24R,25(OH)₂D₃ showed identical LC-MS spectra with those of the 24R,25(OH)₂-3-epi-D₃. It was demonstrated that the molecular weight of the metabolite M was 416 and thus the metabolite M was considered to be a diastereomer of 24R,25(OH)₂D₃. From the results of HPLC, ¹H-NMR and LC-MS analyses, metabolite M could be assigned as 24R,25(OH)₂-3-epi-D₃, the C-3 epimer of 24R,25(OH)₂D₃.

Production of 24R,25(OH)₂-3-epi-D₃ and 1 α ,25(OH)₂-3-

Table 1. ^1H chemical shifts and coupling constants of $24\text{R},25(\text{OH})_2\text{D}_3$ metabolite.

Compound	H-3	H-6	H-7	H-18	H-19	H-21	H-24	H-26,27
$24\text{R},25(\text{OH})_2\text{D}_3$	3.93 (1H, m)	6.21 (1H, d, $J=11.0$)	6.01 (1H, d, $J=11$)	0.53 (3H, s)	4.80 (1H, m) 5.03 (1H, m)	0.92 (3H, d, $J=6.5$)	3.32 (1H, td, $J=4, 8$)	1.15 (3H, s) 1.20 (3H, s)
$24\text{R},25(\text{OH})_2\text{-3-epi-D}_3$	3.86 (1H, m)	6.21 (1H, d, $J=11.0$)	6.02 (1H, d, $J=11$)	0.54 (3H, s)	4.82 (1H, m) 5.04 (1H, m)	0.92 (3H, d, $J=6.5$)	3.31 (1H, td, $J=4, 8$)	1.15 (3H, s) 1.20 (3H, s)
Metabolite M	3.86 (1H, m)	6.21 (1H, d, $J=11.0$)	6.02 (1H, d, $J=11$)	0.54 (3H, s)	4.82 (1H, m) 5.04 (1H, m)	0.92 (3H, d, $J=6.5$)	3.31 (1H, td, $J=4, 8$)	1.15 (3H, s) 1.20 (3H, s)

Chemical shifts and coupling constants are expressed in ppm and Hz, respectively.

Fig. 2. LC-MS spectra of authentic $24\text{R},25(\text{OH})_2\text{D}_3$, $24\text{R},25(\text{OH})_2\text{-3-epi-D}_3$ and metabolite M. Upper: Synthetic standard of $24\text{R},25(\text{OH})_2\text{D}_3$, retention time 4.31 min. Middle: Synthetic standard of $24\text{R},25(\text{OH})_2\text{-3-epi-D}_3$, retention time 4.31 min. Lower: Metabolite M, retention time 4.31 min.

epi-D₃ by UMR 106 cells, Caco-2 cells and LLC-PK₁ cells

The production of both $24\text{R},25(\text{OH})_2\text{-3-epi-D}_3$ and $1\alpha,25(\text{OH})_2\text{-3-epi-D}_3$ was examined in UMR 106 cells, Caco-2 cells and LLC-PK₁ cells incubated with $10\ \mu\text{M}$ of either $24\text{R},25(\text{OH})_2\text{D}_3$ or $1\alpha,25(\text{OH})_2\text{D}_3$. Figures 3A and 3B show the amounts of $24\text{R},25(\text{OH})_2\text{-3-epi-D}_3$ and $1\alpha,25(\text{OH})_2\text{-3-epi-D}_3$ produced from $24\text{R},25(\text{OH})_2\text{D}_3$ and $1\alpha,25(\text{OH})_2\text{D}_3$ respectively, by various cells. It can be seen that the production of $24\text{R},25(\text{OH})_2\text{-3-epi-D}_3$ is lower than that of $1\alpha,25(\text{OH})_2\text{-3-epi-D}_3$ in all the cells studied.

Biological activities of 24R,25(OH)2-3-epi-D3

Biological activities of $24\text{R},25(\text{OH})_2\text{-3-epi-D}_3$ were compared with those of $24\text{R},25(\text{OH})_2\text{D}_3$, $1\alpha,25(\text{OH})_2\text{D}_3$ and $1\alpha,25(\text{OH})_2\text{-3-epi-D}_3$ and the results were summarized in Table 2. When the VDR binding affinity of $24\text{R},25(\text{OH})_2\text{-3-epi-D}_3$ was tested in a competitive binding assay, it was 60 times less potent than $24\text{R},25(\text{OH})_2\text{D}_3$. In contrast, $24\text{R},25(\text{OH})_2\text{-3-epi-D}_3$ and $24\text{R},25(\text{OH})_2\text{D}_3$ showed DBP binding affinity to a similar extent. $24\text{R},25(\text{OH})_2\text{-3-epi-D}_3$ is almost inactive in inhibiting proliferation and in inducing differentiation of HL-60 cells.

DISCUSSION

In the present study we report the conversion of $24\text{R},25(\text{OH})_2\text{D}_3$ into a novel less polar metabolite, $24\text{R},25(\text{OH})_2\text{-3-epi-D}_3$ in UMR 106 cells. The identity

of this novel metabolite of 24R,25(OH)₂D₃ as 24R,25(OH)₂-3-epi-D₃ was confirmed by co-migration with authentic standard on HPLC, ¹H-NMR and LC-MS analysis. Our further studies revealed that Caco-2 and LLC-PK₁ cells also possessed the ability to convert 24R,25(OH)₂D₃ and 1α,25(OH)₂D₃ into 24R,25(OH)₂-3-epi-D₃ and 1α,25(OH)₂-3-epi-D₃, respectively. Thus, the C-3 epimerization of the A-ring appears to be a common metabolic pathway for the two major metabolites of vitamin D₃ in these cells.

1α,25(OH)₂-3-epi-D₃, when compared to

Fig. 3. Production of 24R,25(OH)₂-3-epi-D₃ and 1α,25(OH)₂-3-epi-D₃ by UMR 106 cells, Caco-2 cells and LLC-PK₁ cells incubated with 10 μM of 24R,25(OH)₂D₃ or 1α,25(OH)₂D₃ for 48 h. A: 24R,25(OH)₂D₃. B: 1α,25(OH)₂D₃. The results are expressed as the total amount of product formed in nmol/plate/48 h and represent the mean ± SE of three experiments.

1α,25(OH)₂D₃, was found to possess minimal activity in stimulating intestinal calcium absorption and mobilizing calcium from bone (20). Based on this study, it has been proposed that the C-3 epimerization pathway plays an important role in the inactivation of 1α,25(OH)₂D₃. The C-3 epimerization pathway is also expressed in some cell lines which do not express the C-24 oxidation pathway, indicating that this pathway is an alternate pathway for the inactivation of 1α,25(OH)₂D₃ in these cells (16). However, this hypothesis does not seem to be true in all situations. For example 1α,25(OH)₂-3-epi-D₃ has been shown to retain significant in vitro biological activities in some target cells. 1α,25(OH)₂-3-epi-D₃ was equipotent to its parent 1α,25(OH)₂D₃ in inhibiting keratinocyte growth (20) and in suppressing the secretion of parathyroid hormone in bovine parathyroid cells (17). The higher metabolic stability of 1α,25(OH)₂-3-epi-D₃, when compared to that of 1α,25(OH)₂D₃, has been proposed by us as a possible explanation for the significant in vitro biological activities of 1α,25(OH)₂-3-epi-D₃, in spite of its low binding affinity to the VDR (17). Furthermore in a recent study, it was reported that C-3 epimers of 1α,25(OH)₂D₃ and its synthetic analogs were found to be selective in inducing apoptosis (25, 26). Thus, a careful evaluation of the biological activities of the C-3 epi compounds is needed to fully understand the role of the C-3 epimerization pathway. It is known that 24R,25(OH)₂D₃ lacks affinity to VDR, and as a result exhibits minimal genomic action on vitamin D target genes (27). In the present study, we show that 24R,25(OH)₂-3-epi-D₃ like its parent, 24R,25(OH)₂D₃, is totally inactive in terms of its binding to VDR, and as a result is inactive in inhibiting proliferation and in inducing differentiation of HL-60 cells (Table 2). It has been reported that 24R,25(OH)₂D₃ exhibits non-genomic actions including changes in membrane fluidity (28) and calcium influx (29), turnover of phospholipids (30) and activation of protein kinase C (31). At present, studies are in progress to examine these non-genomic effects of 24R,25(OH)₂-3-epi-D₃.

Table 2. Biological activity of 1α,25(OH)₂D₃, 1α,25(OH)₂-3-epi-D₃, 24R,25(OH)₂D₃ and 24R,25(OH)₂-3-epi-D₃.

	VDR binding affinity ¹	DBP binding affinity ²	Anti-proliferative activity ³	Differentiation inducing activity ⁴
1α,25(OH) ₂ D ₃	100	100	100	100
1α,25(OH) ₂ -3-epi-D ₃	2.1	46	29	9.4
24R,25(OH) ₂ D ₃	0.03	7,182	3.9	0.9
24R,25(OH) ₂ -3-epi-D ₃	0.0005	4,364	<1.5	<0.3

¹ The values represent the relative binding affinity to 1α,25(OH)₂D₃ (expressed as 100), calculated from the concentration of each compound needed to achieve 50% displacement of [³H]-1α,25(OH)₂D₃ from VDR (the concentration of 1α,25(OH)₂D₃ needed to achieve 50% displacement: 9.3×10⁻¹¹ M). ² The values represent the relative binding affinity to 1α,25(OH)₂D₃ (expressed as 100), calculated from the concentration of each compound needed to achieve 50% displacement of [³H]-25(OH)D₃ from DBP (the concentration of 25(OH)D₃ needed to achieve 50% displacement: 8.6×10⁻⁸ M). ³ The values represent the relative anti-proliferative activity to 1α,25(OH)₂D₃ (expressed as 100), calculated from the concentration of each compound needed to achieve 50% 1α,25(OH)₂D₃-induced G₀-G₁ arrest at 10⁻⁷ M (EC₅₀ of 1α,25(OH)₂D₃: 1.5×10⁻⁸ M). ⁴ The values represent the relative differentiation inducing activity to 1α,25(OH)₂D₃ (expressed as 100), calculated from the concentration of each compound needed to achieve 50% 1α,25(OH)₂D₃-induced CD11b expression at 10⁻⁷ M (EC₅₀ of 1α,25(OH)₂D₃: 3.4×10⁻⁹ M).

The nature of the enzyme(s) responsible for the C-3 epimerization is not fully understood. The conversion of $24R,25(OH)_2D_3$ into $24R,25(OH)_2$ -3-epi- D_3 in the present study indicates that the C-1 α hydroxyl group is not a pre-requisite for C-3 epimerization. Also, it remains unclear whether the enzyme(s) involved in the C-3 epimerization of $24R,25(OH)_2D_3$ and $1\alpha,25(OH)_2D_3$ are the same. It is now well established that $1\alpha,25(OH)_2D_3$ -24-hydroxylase (CYP24) performs the various steps of the C-24 oxidation pathway of $1\alpha,25(OH)_2D_3$ indicating that it is a multicatalytic enzyme (32, 33). It was, however, reported that the C-3 epimerization of $1\alpha,25(OH)_2D_3$ was observed in ROS17/2.8 cells that were known to be CYP24-negative cells and in addition, the conversion of $1\alpha,25(OH)_2D_3$ into its C-3 epimer was not inhibited by cytochrom P450 enzyme inhibitors. Thus, CYP24 may not be a candidate enzyme for C-3 epimerization. It has been also reported that 7α -hydroxycholesterol is converted to its C-3 epimer via generation of C-3 oxygenated intermediary metabolites by at least three enzymes (34–38). If these enzymes catalyze the C-3 epimerization of $24R,25(OH)_2D_3$ and $1\alpha,25(OH)_2D_3$, then, their C-3 oxygenated intermediary metabolites should be generated by the cells. As our major concern in the present study was to identify the C-3 epimers of $24R,25(OH)_2D_3$ and $1\alpha,25(OH)_2D_3$ and evaluate their in vitro biological activities, careful examination on the generation of the above intermediary metabolites was not performed and the mechanism of the C-3 epimerization is now under investigation in our laboratory.

It is interesting to note that the production of the C-3 epimer was much greater in $1\alpha,25(OH)_2D_3$ than $24R,25(OH)_2D_3$ in all cell lines tested, suggesting that $1\alpha,25(OH)_2D_3$ may induce the C-3 epimerase expression through binding to VDR, or $1\alpha,25(OH)_2D_3$ may be a high affinity substrate for the C-3 epimerase. Bischof et al. (15) have reported that the activity of the C-3 epimerization of $1\alpha,25(OH)_2D_3$ was completely independent of $1\alpha,25(OH)_2D_3$ -induction, but highly dependent on differentiation and maturation in Caco-2 cells. Thus, the above hypothesis that $1\alpha,25(OH)_2D_3$ induced the C-3 epimerase expression and exhibited high activity of the 3-epimerization can be excluded. The other hypothesis of the difference of the substrate specificity for the C-3 epimerase is probably more realistic if the enzyme recognizes stereochemically not only the orientation of the 3α -hydroxyl group but also the structure of the side chain and A-ring of the vitamin D metabolites. We are currently isolating the putative enzyme(s) from the UMR 106 cells and characterizing the properties of the enzyme(s). As expected from the report of Bischof et al. (15), the activity of the C-3 epimerization is affected by many factors, such as differentiation and proliferation stages of the cells, stability of the C-3 epimers in the medium or the cells and so forth. To address these issues, studies are in progress to characterize the enzyme(s) responsible for the C-3 epimerization of $24R,25(OH)_2D_3$ and $1\alpha,25(OH)_2D_3$.

In summary, we report for the first time the conver-

sion of $24R,25(OH)_2D_3$ into $24R,25(OH)_2$ -3-epi- D_3 in three different cell lines. The identity of $24R,25(OH)_2$ -3-epi- D_3 was clearly confirmed by HPLC, 1H -NMR and LC-MS analyses. $24R,25(OH)_2$ -3-epi- D_3 was found to be totally inactive in terms of its binding to VDR and its ability to inhibit proliferation and induce differentiation of HL-60 cells. Thus, our study indicates that C-3 epimerization pathway is common to both $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ and may play an important role in the regulation of the concentration of these two major vitamin D_3 metabolites in target cells.

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