Studies on Vitamin D Levels in Biological Samples and Novel Metabolites of Vitamin D

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ABBREVIATIONS

ADR	adorenodoxin reductase
ADT	androsterone
ADX	adrenodoxin
AI	adequate intake
AIB	amplified in breast cancer
ANOVA	analysis of variance
BUA	broadband ultrasound attenuation
BMI	body mass index
CE	collision energy
CLIA	chemiluminescence immunoassay
COSY	correlation spectroscopy
CPBA	competitive protein binding assay
CYP	cytochorome P450
CXP	collision cell exit potential
D_2	vitamin D_2
D_3	vitamin D_3
$1\alpha(OH)D_3$	1α-hydroxyvitamin D_3
$1\alpha,20(OH)_2D_3$	1α,20-dihydroxyvitamn D_3
$1\alpha,24,25(OH)_3D_3$	1α,24,25-trihydroxyvitamin D_3
$1\alpha,25(OH)_2D_2$	1α,25-dihydroxyvitamin D_2
$1\alpha,25(OH)_2D_3$	1α,25-dihydroxyvitamin D_3
$20\text{-oxo-}1\alpha(OH)D_3$	hexanor-20-oxo-1α-hydroxyvitamin D_3
24(OH)OCT	24-hydroxy-OCT
$24,25(OH)_2D_3$	24,25-dihydroxyvitamin D_3
$24,25(OH)_2D_3$	24,25-dihydroxyvitamin D_2
$24\text{-ene-22-oxa-}1\alpha(OH)D_3$	24-ene-22-oxa-1α-hydroxyvitamin D_3
25(OH)D	25-hydroxyvitamin D [$25(OH)D_3$+$25(OH)D_2$]
$25(OH)D_2$	25-hydroxyvitamin D_2
$25(OH)D_3$	25-hydroxyvitamin D_3
$25\text{-ene-22-oxa-}1\alpha(OH)D_3$	25ene-22-oxa-1α-hydroxyvitamin D_3
d	doublet
DBP	vitamin D binding protein

dd	doublet of doublets
ddd	doublet of doublets of doublets
DMEM	Dulbecco's modified Eagle's medium
DMEQ-TAD	4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione
DNA	deoxyribonucleic acid
DP	declustering potential
DRIs	dietary reference Intakes
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid, dihydrate
EIA	enzyme immunoassay
ESI	electrospray ionization
FCS	fetal calf serum
FFQ	food-frequency questionnaire
FP	focusing potential
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hertz	Hz
HIM	hexane/2-propanol/methanol
HPLC	high-performance liquid chromatography
HPTLC	high performance thin layer chromatography
HS	high school
JHS	junior high school
Km	Michaelis constant
LC-APCI/MS/MS	liquid chromatography-atmospheric pressure chemical ionization/tandem mass spectrometry
LC-MS	liquid chromatography mass spectrometry
LC-MS/MS	liquid chromatography-tandem mass spectrometry
m	multiplet
MEM	minimum essential medium
MK	menaquinone, vitamin K_2
MRM	multiple reaction monitoring
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NMR	nuclear magnetic resonance

NOESY	nuclear Overhauser effect correlated spectroscopy
OC	osteocalcin
OCT	22-oxacalcitriol, maxacalcitriol
26(OH)OCT	26-hydroxy-OCT
PBS (−)	phosphate-buffered saline without Ca, Mg
pH	hydrogen ion concentration
PK	phylloquinone, vitamin K_1
PTH	parathyroid hormone
QUS	quantitative ultrasound
R.T.	retention time
RDAs	recommended daily allowances
RE	retinol
RIA	radio immunoassay
RoDHs	retinol dehydrogenases
S	substrate concentration
s	singlet
SD	standard deviation
SDR	short chain alcohol dehydrogenase family
SE	standard errors
sl	singlet like
SOS	speed of sound
SRC	steroid receptor coactivator
t	triplet
td	triplet of doublets
TIF	transcriptional intermediary factor
α-Toc	α-Tocopherol
UDP	uridine diphosphate
UV	ultraviolet
V	velocity
VDR	vitamin D receptor
VDRE	vitamin D-responsive element
Vmax	maximum velocity
WHO	World Health Organization
1D	one-dimensional
1st HS	1st grade of high school

1st JHS	1st grade of junior high school
2D	two-dimensional
3(α→β)-HSE	3(α→β)-hydroxysteroid epimerase
3α-HSD	3α-hydroxysteroid dehydrogenase
3rd HS	3rd grade of high school

GENERAL INTRODUCTION

Vitamin D is well known to play an important role in calcium homeostasis and bone metabolism. Vitamin D may also have several nonskeletal functions such as immune system [1, 2], reproduction [3, 4] and cancer prevention [5, 6]. Vitamin D_3 (D_3) is synthesized in the skin from 7-dehydrocholesterol by exposure to sunlight. D_3 is metabolized to 25-hydroxyvitamin D_3 [$25(OH)D_3$] in the liver and subsequently to 1α,25-dihydroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$] or 24,25-dihydroxyvitamin D_3 [$24,25(OH)_2D_3$] in the kidney [7] (Fig.1). $1\alpha,25(OH)_2D_3$ is known to be the most active form and exert its biological actions through binding with the nuclear vitamin D receptor (VDR). $24,25(OH)_2D_3$ is considered to be inactive form of vitamin D_3 although there are several reports demonstrating its potentials increasing bone volume and strength at pharmacological doses [8, 9]. $25(OH)D_3$ is most abundant circulating metabolite of vitamin D and good indicator of the cumulative effects of exposure to sunlight and dietary intake of D_3. Vitamin D_2 (D_2), which is the major naturally occurring form in plants, is also metabolized to 25-hydroxyvitamin D_2 [$25(OH)D_2$], 1α,25-dihydroxyvitamin D_2 [$1\alpha,25(OH)_2D_2$] and 24,25-dihydroxyvitamin D_2 [$24,25(OH)_2D_3$] in a similar fashion. To date, in addition to these metabolites, many other vitamin D_3 metabolites have been chemically characterized, and another unidentified metabolites are assumed to exist.

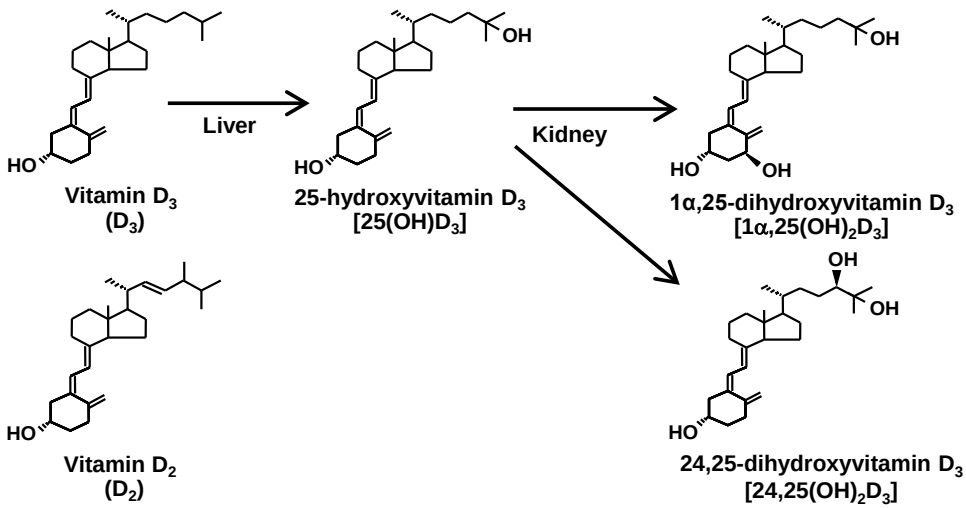

Fig. 1 Structure and metabolic pathway of vitamin D

In recent epidemiological study, lower serum 25-hydroxyvitamin D [25(OH)D, $25(OH)D_3$+$25(OH)D_2$] level has been reported to be associated with a higher risk of Alzheimer disease [10], type 2 diabetes [11] and acute respiratory tract infections in newborns [12] in addition to bone fracture risk [13, 14]. These finding suggest that vitamin D is an important nutrient at each stage in life from infancy to old age. Thus, it is important to measure the concentration of vitamin D metabolites and define reference value in biological samples such as serum and breast milk. In addition, it is also important to identify unknown metabolites of vitamin D and to examine their biological activity in explanation of wide-ranging biological activities of vitamin D and their regulation mechanism.

In Part 1, we measured vitamin D levels in biological samples and showed reference value of them. In Part 1, Chapter 1, first, a high-sensitive determination method of vitamin D and other fat-soluble vitamins in human breast milk using liquid chromatography-atmospheric pressure chemical ionization/tandem mass spectrometry (LC-APCI/MS/MS) was developed. Then we applied this method to breast milk of Japanese lactating mothers to estimate an infant's intake of vitamin D and other fat-soluble vitamins. In Part 1, Chapter 2, we measured serum level of 25(OH)D in a large group consisting of over 1300 Japanese adolescents using an automated competitive chemiluminescence immunoassay (CLIA) [15]. The sex differences in the association between serum 25(OH)D concentration and serum parathyroid hormone (PTH) concentration or vitamin D intake were also examined.

In Part 2, we examined metabolism of native vitamin D and representative synthetic analog and identified novel metabolites including C-3 epimers and C-25 dehydrates. In Part 2, Chapter 1, we demonstrated that $25(OH)D_3$ which is the most abundant metabolites of vitamin D is metabolized to its C-3 epimer as well as $1\alpha,25(OH)_2D_3$ and $24,25(OH)_2D_3$. In addition, further metabolism and biological activity of C-3 epimers were examined. In Part 2, Chapter 2, we identified novel three less polar metabolites of representative synthetic analog of vitamin D, 22-oxacalcitriol (OCT, maxacalcitriol) as 3-epi-OCT and two C-25

dehydrates and assessed the biological activity of them. Moreover, in Part 2, Chapter 3, we measured C-3 epimerization activity in subcellular fractions prepared from cultured cells and investigated the basic properties of the enzyme responsible for the C-3 epimerization.

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PART 1

Analysis of vitamin D levels in biological samples

CAPTER 1

Quantitative analysis of vitamin D and other fat-soluble vitamins in human breast milk

Introduction

International agencies including World Health Organization (WHO) and health organizations of various countries recommend breastfeeding as the preferred method of infant feeding for the first several months of life [1]. There is no doubt that breastfeeding is beneficial to the relationship between mother and infant. According to WHO, exclusive breast-feeding is recommended up to six months of age, with continued breast-feeding along with appropriate complementary foods up to two years of age of beyond. Term infants nursed by nutritionally adequate mothers are provided with sufficient energy and the proper profile of nutrients to support normal growth and development during the first six months except for vitamins D and K in the immediate newborn period [2, 3].

Exclusive breastfeeding is a risk factor for rickets in infants. There have been reports of clinical rickets in breastfed infants, especially nursed by mothers who restrict their intake of vitamin D-rich foods (i.e. strict vegetarians) [4]. Also, in countries where climate or custom lead to low levels of exposure of the child or the mother to sunlight, infant serum concentrations of 25(OH)D may be sub-optimal [5, 6]. In previous reports, the concentrations of D_3, $25(OH)D_3$, $24,25(OH)_2D_3$ and $1\alpha,25(OH)_2D_3$ in human breast milk were 0.03–0.12, 0.28–0.57, 0.04–0.28 and 0.005–0.02 ng/mL, respectively [7–10]. These results indicate that most anti-rachitic activity is caused by $25(OH)D_3$, and the transfer of vitamin D and its metabolites from plasma to milk is limited.

Vitamin K is also a possible problem for breast-fed infants. Human breast milk contains only a low concentration of vitamin K and there is strong evidence of increased incidence of late haemorrhagic disease in breastfed infants [11]. It was reported that the vitamin K_1 (phylloquinone, PK) concentration of human breast milk ranges from 1 to 9 ng/mL [12, 13]; however, there are few reports associated with other important vitamin K derivatives,

vitamin K_2 (menaquinone, MK), contents [14]. Regional or individual nutritional problems with Vitamin A and E in breast-fed infants are also reported. In the developing countries, subclinical vitamin A deficiency has been observed in infants fed breast milk. The vitamin A content of milk in poorer populations in developing countries such as India, Ceylon and Jordan, where vitamin A intake is marginal, is lower than in North America and Europe [15]. Meanwhile, there has been no report of problems with vitamin E for breast-fed infants.

In this manner, surveys of the concentrations of vitamin D and other fat-soluble vitamins in human breast milk have important implications for the promotion of breast-feeding. However, there are several problems in sensitivity, specificity and accuracy with determination of fat-soluble vitamins in breast milk. Especially, it is difficult to determine vitamin D and metabolites using standard assay methods such as high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [16], competitive protein binding assay (CPBA) [17], radio immunoassay (RIA) [18] and enzyme immunoassay (EIA) [19], because concentrations of them are markedly low. In this study, we have developed a high-sensitive quantification method of vitamin D and other fat-soluble vitamins in human breast milk using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and applied to a nutrition survey for lactating mothers.

Materials and methods

Materials.

D_3, D_2, 25(OH)D_3 and 25(OH)D_2 were obtained from Solvay Pharmaceuticals B.V. (Veenendaal, The Netherlands). Retinyl palmitate and β-Carotene were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). α-Tocopherol (α-Toc), PK, MK-4 and MK-7 were kindly provided by Eisai Pharmaceuticals (Tokyo, Japan). Four internal standards, d_6-25-(OH)D_3, [$^{18}O_2$]-PK, [$^{18}O_2$]-MK-4 and [$^{18}O_2$]-MK-7 were synthesized as described in our previous reports [20, 21]. D_6-retinyl acetate, d_6-β-carotene, d_7-D_3, and d_6-α-Toc were synthesized by Dr. Suhara. The chemical structures of the internal standards are shown in Fig. 1. Derivatization reagent, 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of Retinol and d_6-Retinol.

Retinol (RE) and d_6-RE solutions were prepared by saponifying retinyl palmitate and d_6-retinyl acetate before use. 40 μg of retinyl palmitate and d_6-retinyl acetate were dissolved in 1.5 mL of pyrogallol-ethanol (7 %, w/v). After the addition of 0.5 mL of NaCl solution (1 %, w/v) and 0.8 mL of KOH solution (60 %, w/v), the mixture was incubated at 70 ºC for 30 min. RE and d_6-RE were extracted with hexane-diethyl ether (90:10, v/v), evaporated under reduced pressure, and the residue was dissolved with 2-propanol.

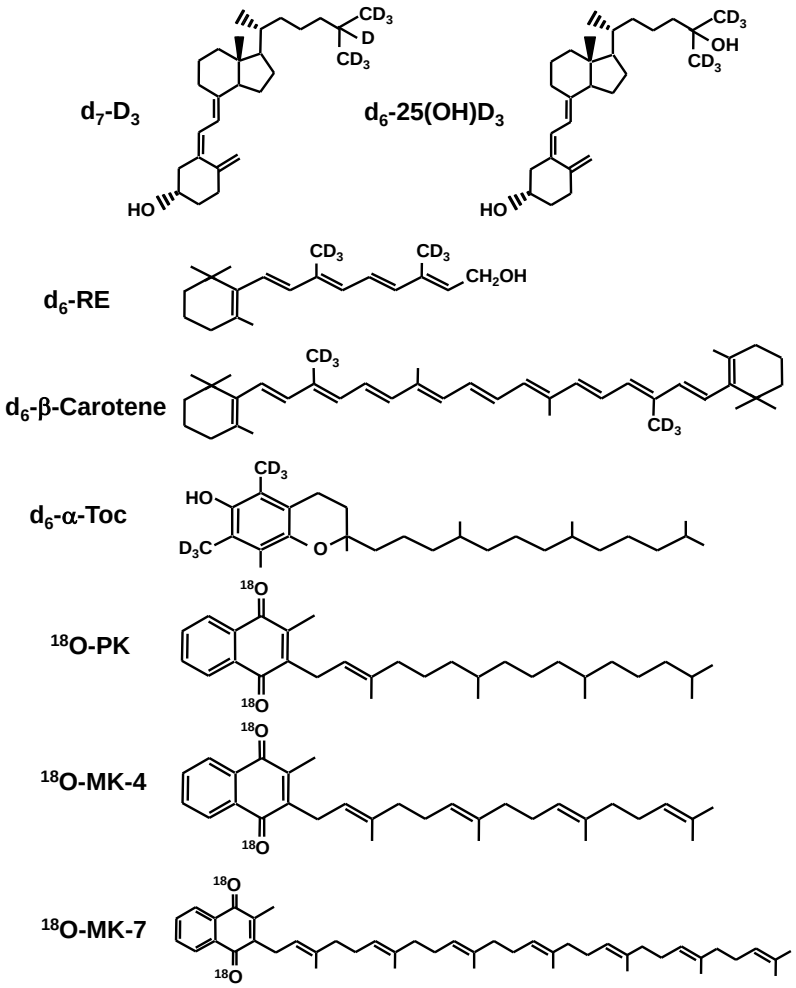

Fig.1 Chemical structures of internal standards.

Concentrations of RE and d_6-RE were determined spectrophotometrically using a molar extinction coefficient, ε = 52,480. The purity was checked by HPLC consisting of an LC-10AD$_{VP}$ pump, a SIL-10AD$_{VP}$ auto injector, a CTO-10AD$_{VP}$ column oven set to 35 ºC, and an RF-10A$_{XL}$ fluorescence detector set to an excitation wavelength of 340 nm and an emission wavelength of 460 nm (Shimadzu, Kyoto, Japan). Separation was performed on a Capcellpak C18 UG120 (4.6 × 250 mm, Shiseido Co. Ltd., Tokyo, Japan) eluted with ethanol: H_2O (95:5) at a flow rate of 0.4 mL/min.

Sample Collections.

Human breast milk samples were collected from March 2005 to October 2006 from 82 lactating mothers aged 18–39 y (30.8 ± 4.5 y) at 3–265 d (49.1 ± 57.6 d) post-partum living in Japan. Written informed consent was obtained from each subject prior to enrollment in this study according to the conditions of the Helsinki Declaration and approved by the ethics committee of Kobe Pharmaceutical University. Approximately 50 mL of human breast milk was collected by manual expression at an intermediate time during suckling and immediately frozen at −20 ºC. Before extraction of vitamin D and other fat-soluble vitamins, frozen breast milk was thawed and sonicated in ice water twice for 15 min. For the developmental work on the assay, pooled human breast milk prepared by mixing breast milk from seven healthy mothers was used.

Extraction of Vitamin D, Vitamin A, β-Carotene and Vitamin E.

10.0 mL of breast milk samples was placed in a 50-mL screw-top vial. After the addition of 50 μL of internal standard solution [d_7-D_3, d_6-25(OH)D_3, d_6-RE, d_6-β-carotene and d_6-α-Toc, 50 ng/50 μL ethanol each], 20 mL of pyrogallol-ethanol (7 %, w/v), 6 mL of NaCl solution (1 %, w/v) and 10 mL of KOH solution (60 %, w/v), the mixture was incubated at 70 ºC for 60 min. Then, the mixture was transferred to a 200-mL of separating funnel containing 38 mL of NaCl solution (1 %, w/v) and vitamin D, vitamin A, β-carotene and vitamin E were extracted twice with 30 mL of hexane-ethyl acetate (9:1, v/v), washed with water, and dehydrated with Na_2SO_4. The eluate was evaporated under reduced pressure, and the residue was dissolved with 2.5 mL of hexane:ethyl acetate (9:1, v/v). For determination of D_3, D_2, 25(OH)D_3 and 25(OH)D_2, 1.5 mL of 2.5 mL was concentrated and purified by normal phase HPLC. HPLC was carried out using a model 600 pump and a model 996

photodiode array detector (Waters Associates, Milford, MA). Elution was performed on a Zorbax SIL column (4.6 × 250 mm, Agilent Technologies, Santa Clara, CA), using hexane:2-propanol:methanol (88:10:2, v/v/v), at a flow rate of 1.0 mL/min. After the eluates corresponding to D_3 and D_2 (D fraction, 3.5-5.0 min) and 25(OH)D_3 and 25(OH)D_2 (25D fraction, 5.0-8.0 min) were collected, DMEQ-TAD derivatization was performed by described below. For the determination of RE, β-carotene and α-Toc, another 1.0 mL was evaporated, and the residue was dissolved with 100 μL of ethanol, 50 μL of which was directly subjected to LC-MS/MS.

DMEQ-TAD Derivatization of D and 25(OH)D Fractions.

DMEQ-TAD derivatization was performed according to the method of Higashi et al. [22]. D and 25(OH)D fractions were dried and then dissolved in 150 μL of ethyl acetate containing DMEQ-TAD (60 μg). The mixture was kept at room temperature for 30 min, then an additional reagent (60 μg/150 μL of ethyl acetate) was added and the entire mixture was further kept at room temperature for 1 h. After the addition of 1.5 mL of ethanol to decompose excess reagent, the solvent was evaporated and the residue was dissolved in 100 μL of acetonitrile, 30 μL of which was subjected to LC-MS/MS.

Extraction of Vitamin K Derivatives.

3.0 mL of breast milk samples was placed in a 50-mL screw-top vial. After the addition of internal standard solution ([$^{18}O_2$]-PK, [$^{18}O_2$]-MK-4 and [$^{18}O_2$]-MK-7, 25 ng/25 μL ethanol each), 12 mL of phosphate buffer (pH 7.7) and 0.3 g of lipase, the mixture was incubated at 37 ºC for 90 min with stirring. Then, 12 mL of ethanol was added and vitamin K derivatives were extracted twice with 12 mL of hexane. The mixture was shaken for 5

min before centrifuging at 1,940 × g for 5 min. The extracts were combined and evaporated under reduced pressure, and the residue was dissolved with 3 mL of hexane. The resultant extract was passed through a Sep-Pak Vac silica cartridge (Waters, Milford, MA, USA) that was washed with 10 mL of hexane. Vitamin K derivatives were eluted with 5 mL of hexane-diethyl ether (97:3). The eluate was evaporated under reduced pressure, and the residue was dissolved with 200 μL of ethanol, 50 μL of which was subjected to LC-MS/MS.

LC-MS/MS Analysis.

The HPLC system consisted of a SCL-10ADvp system controller, two LC-10ADvp pumps, a DGC-14A automatic solvent degasser, a SIL-10ADvp auto injector, and a CTO-10ADvp column oven set to 35 ºC (Shimadzu). The HPLC system was coupled to an API 3000 triple-quadrupole tandem mass spectrometer (Applied Biosystems/ MDS SCIEX, Foster City, CA, USA) equipped with an atmospheric pressure chemical ionization (APCI) source. Analyst (Ver. 1. 3. 2; Applied Biosystems/MDS SCIEX) was used for data acquisition and analysis. Separations were performed on a Capcellpak C18 UG120 (4.6 × 250 mm). For the determination of DMEQ-TAD derivatives of D and 25(OH)D, a solvent system consisting of a mixture of acetonitrile and H_2O (30:70, v/v) in 5 min and then a linear gradient from 30 to 95 % acetonitrile in 30 min was used (Condition 1). The injection volumes of standard and sample solutions were 30 μL. For the determination of RE, β-carotene, α-Toc and vitamin K derivatives, a solvent system consisting of an isocratic solvent A (methanol:H_2O, 90:10, v/v) in 10 min and then a linear gradient from 0 to 90 % acetonitrile in 30 min was used (Condition 2). The injection volumes of standard and sample solutions were 50 μL. Acquisition settings were optimized by the infusion of a 1 μg/mL solution of each compound at a rate of 20 μL/min as shown in Table 1. Curtain gas (8 psi),

nebulizer gas (8 psi), collision gas (4 psi), nebulizer current (2 μA) and ion source temperature (400 ºC) were identical for all analytes. The mass spectrometer was operated in the positive ion mode. All analytes were detected in the MS/MS-multiple reaction monitoring (MRM) with unit resolution at both Q1 and Q3.

Table 1 Retention times, molecular weights and optimized instrument settings.

Analyte	Retention time (min)	MW	Transitions, m/z		DP[1] (V)	FP[1] (V)	CE[1] (V)	CXP[1] (V)
			Parent ion	Product ion				
Condition 1								
DMEQ-TAD-D_3[2]	36.46	729.9	730.5	468.3	61	200	35	32
DMEQ-TAD-D_2[2]	36.43	742.0	742.6	468.3	56	170	35	34
DMEQ-TAD-d_7-D_3[2]	36.30	737.0	737.6	468.2	56	210	33	8
DMEQ-TAD -25(OH)D_3[2]	22.21	745.9	746.5	468.1	61	210	37	16
DMEQ-TAD -25(OH)D_2[2]	21.92	758.0	758.5	468.2	56	180	37	16
DMEQ-TAD -d_6-25(OH)D_3[2]	22.08	752.0	752.5	468.1	56	190	39	16
Condition 2								
RE	9.73	286.5	269.1	213.4	21	80	19	14
d_6-RE	9.48	292.5	275.2	192.4	16	70	19	14
β-carotene	87.01	536.9	537.6	177.2	31	100	27	12
d_6-β-carotene	86.24	542.9	543.6	180.2	31	110	25	12
α-Toc	38.72	430.7	430.4	165.2	51	180	43	10
d_6-α-Toc	38.44	436.7	436.5	171.2	56	180	41	10
PK	49.15	450.7	451.5	187.1	41	140	33	12
[$^{18}O_2$]-PK	49.13	454.7	455.4	191.2	41	140	33	12
MK-4	32.72	444.7	445.5	187.3	21	80	31	12
[$^{18}O_2$]-MK-4	32.66	448.7	449.4	191.2	26	100	31	12
MK-7	86.74	649.0	649.7	187.2	41	150	47	12
[$^{18}O_2$]-MK-7	86.67	653.0	653.7	191.1	36	130	43	12

[1] DP, declustering potential; FP, focusing potential; CE, collision energy; CXP, collision cell exit potential

[2] The retention times of the derivatives are those of the 6*S*-isomer.

Statistical Analysis.

All statistical analyses were performed using JMP statistical software (version 5.0.1 J: SAS Institute Inc, Cary, NC, USA). For cross-sectional analyses, simple regression analysis was performed.

Results

Chromatography.

LC-MS/MS MRM chromatograms of human breast milk samples after DMEQ-TAD derivatization for the determination of D_3, D_2, 25(OH)D_2 and 25(OH)D_3 in Condition 1 are shown in Fig. 2. In the reaction with DMEQ-TAD, vitamin D compounds produce two C6-epimeric derivatives. In the case of D_3, D_2, 25(OH)D_3 and 25(OH)D_2, the 6S isomer was the main product (6S:6R=3:1). Thus, 6S isomer was used for the determination of each vitamin D compound. The calibration curves of DMEQ-TAD derivatives showed good linearity for D_3 (up to 50 ng/mL, r^2=0.9999), D_2 (up to 50 ng/mL, r^2=1.0000), 25(OH)D_3 (up to 50 ng/mL, r^2=0.9999) and 25(OH)D_2 (up to 50 ng/mL, r^2=0.9991), respectively.

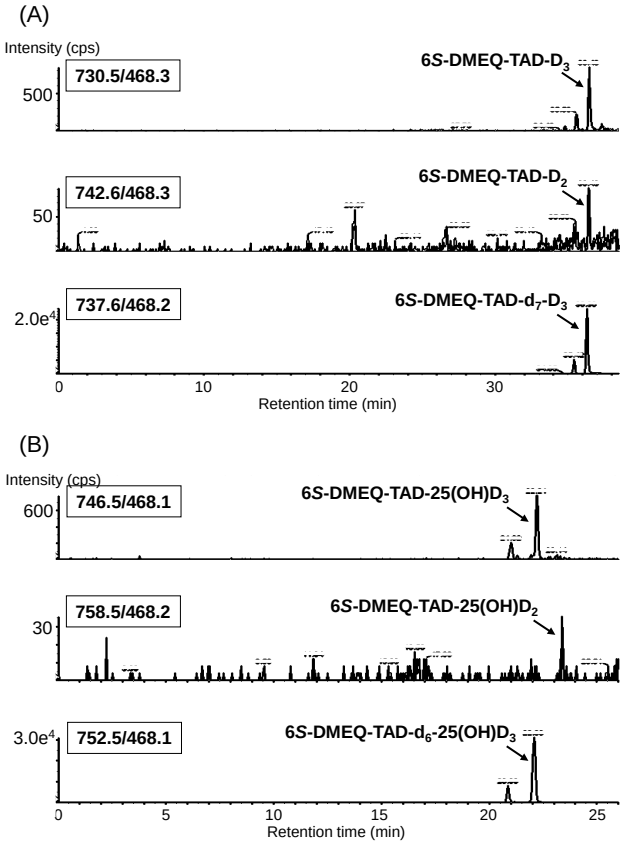

Fig. 2 LC-MS/MS MRM chromatograms of human breast milk sample after DMEQ-TAD derivatization for determination of D_3 and D_2 (A), 25(OH)D_2 and 25(OH)D_3 (B) in Condition 1. The concentrations of D_3, D_2, 25(OH)D_2 and 25(OH)D_3 in this sample are 0.058, 0.005, 0.002 and 0.103 ng/mL, respectively.

MRM chromatograms of human breast milk sample for the determination of RE, β-carotene, α-Toc, PK, MK-4 and MK-7 in Condition 2 are shown in Fig. 3. Under these conditions, all compounds were successfully detected without interruption of co-eluting compounds in breast milk and interference of their internal standards. The calibration curves showed good linearity for RE (up to 62500 ng/mL, r^2=0.9993), β-carotene (up to 2500 ng/mL, r^2=0.9989), α-tocopherol (up to 62500 ng/mL, r^2=0.9998), PK (up to 2500 ng/mL, r^2=1.0000), MK-4 (up to 2500 ng/mL, r^2=0.9998) and MK-7 (up to 2500 ng/mL, r^2=1.0000), respectively.

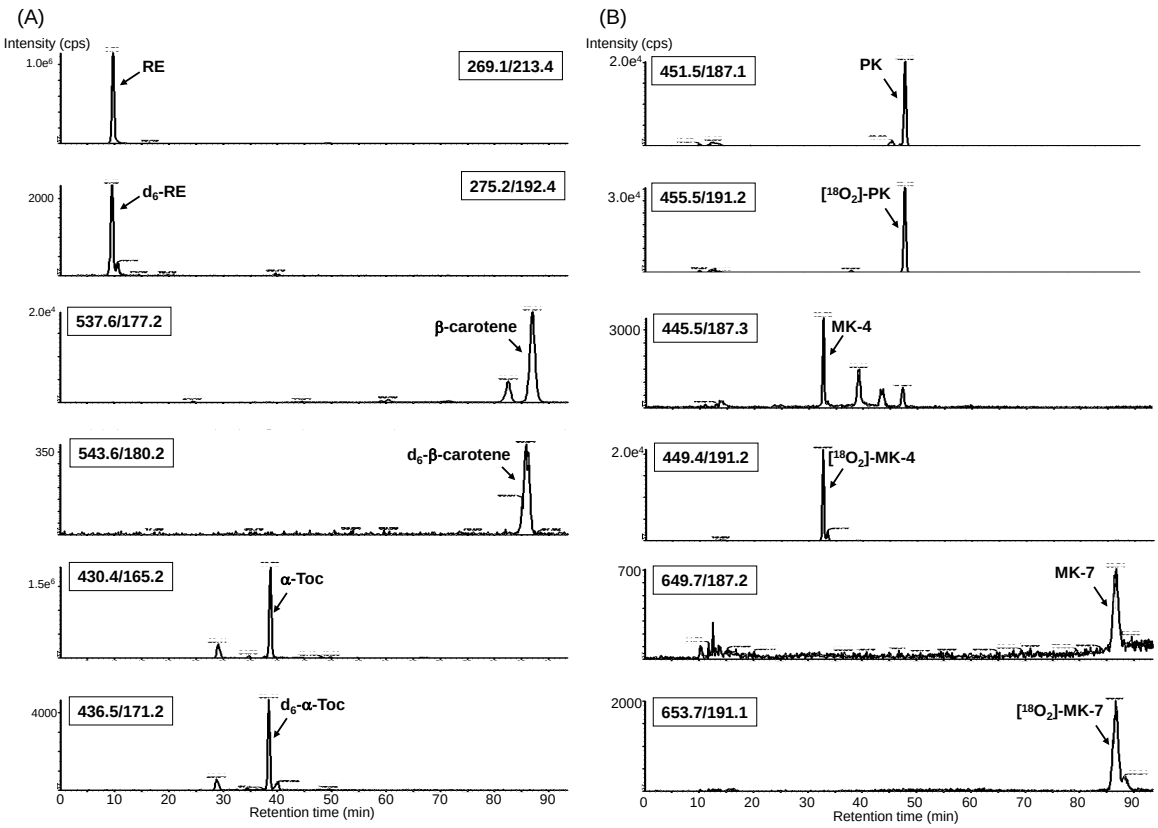

Fig. 3 LC-MS/MS MRM chromatograms of human breast milk sample for determination of RE, β-carotene and α-Toc (A), PK, MK-4 and MK-7 (B) in Condition 2. The concentrations of RE, β-carotene and α-Toc, PK, MK-4 and MK-7 in this sample are 0.244, 0.055 and 2.131 μg/mL, 1.628, 1.462 and 0.344 ng/mL, respectively.

Sensitivity, Recovery and Reproducibility.

The detection limits of D_3, D_2, 25(OH)D_3, 25(OH)D_2, RE, β-carotene, α-Toc, PK, MK-4 and MK-7 based on a signal-to-noise ratio of 3:1, were 1 to 250 pg per injection (Table 2). The recoveries were about 91–105 %. Inter-assay CV values of each vitamin calculated by measurements of pooled human breast milk were 1.9–11.9 %. The sensitivity and overall recovery combined with reproducibility allowed the measurement of fat-soluble vitamins containing vitamins D, A, E and K with 10 mL of breast milk.

Table 2 Accuracy of measurement of fat-soluble vitamins

	Detection limit (pg)	Recovery [1]		Inter-assay	
		Mean ± SD (%)	CV (%)	Mean ± SD (/mL)	CV (%)
D_3	1	97.5±3.8	3.9	0.040±0.002 ng	5.4
D_2	1	105.0±4.7	4.5	0.014±0.002 ng	11.9
25(OH)D_3	2	93.9±3.0	3.1	0.117±0.005 ng	4.0
25(OH)D_2	1	90.9±8.8	9.7	0.006±0.001 ng	9.8
RE	50	104.7±7.5	7.2	0.489±0.029 μg	7.2
β-carotene	250	97.4±7.0	8.2	0.027±0.001 μg	3.8
α-Toc	100	96.3±5.5	5.7	2.839±0.058 μg	2.1
PK	10	97.5±8.3	8.6	0.383±0.014 ng	3.7
MK-4	10	99.4±7.9	8.0	0.206±0.004 ng	1.9
MK-7	80	97.1±5.6	5.7	0.117±0.011 ng	9.6

[1] Calculated by measurements of pooled human breast milk spiked with fat-soluble vitamins: D_3, 20 ng; D_2, 20 ng; 25(OH)D_3, 20 ng; 25(OH)D_2, 20 ng; RE, 20 μg; β-carotene, 0.6 μg; α-Toc, 30 μg; PK, 30 ng; MK-4, 20 ng; MK-7, 10 ng/20 mL of human breast milk.

Concentration of Vitamin D and Other Fat-soluble Vitamins in Human Breast Milk.

This method was applied to breast milk samples obtained from 82 Japanese lactating mothers. The mean concentration of D_3, D_2, 25(OH)D_3 and 25(OH)D_2 were 0.088 (range, 0.010–1.116; median, 0.061) ng/mL, 0.078 (range, 0–1.300; median, 0.021) ng/mL, 0.081 (range, 0.023–0.172; median, 0.078) ng/mL and 0.003 (range, 0–0.012; median, 0.003) ng/mL, respectively. The mean concentration of RE, β-carotene, α-Toc, PK, MK-4 and MK-7 were 0.455 (range, 0.097–1.783; median, 0.406) μg/mL, 0.062 (range, 0.002–0.375; median, 0.045) μg/mL, 5.087 (range, 0.387–35.664; median, 3.590) μg/mL, 3.771 (range, 0.953–12.382; median, 3.481) ng/mL, 1.795 (range, 0.720–4.750; median, 1.611) ng/mL and 1.540 (range, 0.074–15.861; median, 1.001) ng/mL, respectively (Table 3).

Table 3 Concentration of fat-soluble vitamins in human breast milk

		Total (n=82)
		Mean±SD
Age	(y)	30.8±4.5
D_3	(ng/mL)	0.088±0.128
D_2	(ng/mL)	0.078±0.156
25(OH)D_3	(ng/mL)	0.081±0.037
25(OH)D_2	(ng/mL)	0.003±0.002
RE	(μg/mL)	0.455±0.264
β-carotene	(μg/mL)	0.062±0.063
α-Toc	(μg/mL)	5.087±5.042
PK	(ng/mL)	3.771±2.166
MK-4	(ng/mL)	1.795±0.732
MK-7	(ng/mL)	1.540±2.298
Fat[1]	(mg/mL)	28.89±11.65

[1] Measured by Röse-Gotlieb method.

The subjects were stratified into five groups by post-partum days: 0–10, 11–30, 31–90, 91–180 and 181–270 days. The concentrations of many fat-soluble vitamins in human breast milk showed a tendency to decrease as the post-partum days passed (Table 4). The concentrations of 25(OH)D_2, RE, β-carotene, and α-Toc in breast milk in the 0–10 day post-partum group were significantly higher than those of the other groups ($p<0.05$). Fat concentrations were not significantly different between each group.

Table 4 Cross-sectional analyses of fat-soluble vitamins in human breast milk[1]

Post-partum days		0–10 (n=8)	11–30 (n=43)	31–90 (n=18)	91–180 (n=8)	181–270 (n=5)
		Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Age	(y)	27.6±6.3[a]	32.0±3.6[a]	30.3±4.5[a]	30.4±5.6[a]	28.6±4.3[a]
D_3	(ng/mL)	0.075±0.046[a]	0.103±0.169[a]	0.079±0.056[a]	0.075±0.079[a]	0.035±0.016[a]
D_2	(ng/mL)	0.129±0.076[a]	0.073±0.199[a]	0.066±0.084[a]	0.014±0.005[a]	0.181±0.099[a]
25(OH)D_3	(ng/mL)	0.072±0.047[a]	0.085±0.038[a]	0.084±0.034[a]	0.068±0.037[a]	0.073±0.041[a]
25(OH)D_2	(ng/mL)	0.007±0.003[a]	0.003±0.002[b]	0.003±0.002[b]	0.003±0.003[b]	0.003±0.001[b]
RE	(μg/mL)	1.026±0.398[a]	0.418±0.138[b]	0.384±0.145[b]	0.359±0.219[b]	0.267±0.117[b]
β-carotene	(μg/mL)	0.188±0.112[a]	0.059±0.037[b]	0.033±0.023[b]	0.033±0.031[b]	0.043±0.048[b]
α-Toc	(μg/mL)	16.590±9.635[a]	4.079±1.795[b]	3.911±1.798[b]	3.296±1.962[b]	2.454±1.045[b]
PK	(ng/mL)	5.122±2.561[a]	3.938±2.450[a]	3.528±1.454[a]	2.294±1.220[a]	3.409±1.462[a]
MK-4	(ng/mL)	2.561±1.207[a]	1.802±0.664[b]	1.785±0.553[ab]	1.195±0.343[ab]	1.510±0.419[b]
MK-7	(ng/mL)	3.044±2.901[a]	1.675±2.732[a]	0.798±0.746[a]	1.363±1.292[a]	0.917±0.916[a]
Fat[2]	(mg/mL)	24.92±11.55[a]	32.64±11.52[a]	30.24±7.91[a]	21.39±14.12[a]	20.72±10.08[a]

[1] Means in the same row bearing different superscripts differ significantly ($p<0.05$) by Tukey-Kramer HSD test.

[2] Measured by Röse-Gotlieb method.

The relations between the concentrations of each fat-soluble vitamin in breast milk and the other parameters are shown in Table 5. Age of lactating mothers correlated significantly and positively with the concentration of D_3 in breast milk. The concentration of 25(OH)D_3 was positively correlated with β-carotene, PK and fat. The concentration of 25(OH)D_2 was also positively correlated with RE, β-carotene, α-tocopherol and PK. It should be noted that correlations between the concentrations of D_3 and D_2 or 25(OH)D_3 and 25(OH)D_2 were observed. However, correlations between D and 25(OH)D were not found.

Table 5 Relation between concentrations of vitamin D and metabolites in human milk and the other parameters[1]

	D_3		D_2		25(OH)D_3		25(OH)D_2	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Age	0.2188	**0.0483**	0.1941	0.0806	0.2004	0.0710	-0.2127	0.0550
Post-partum days	-0.1187	0.2881	0.0745	0.5058	-0.1021	0.3615	-0.0462	0.6806
D_3	-	-	-	-	-	-	-	-
D_2	0.7984	**<.0001**	-	-	-	-	-	-
25(OH)D_3	0.1490	0.1815	-0.0967	0.3875	-	-	-	-
25(OH)D_2	-0.0889	0.4269	-0.0824	0.4617	0.2748	**0.0125**	-	-
RE	0.0294	0.7931	-0.0017	0.9879	0.0548	0.6248	0.2794	**0.0110**
β-carotene	0.0349	0.7554	0.0602	0.5908	0.2208	**0.0462**	0.4132	**0.0001**
α-Toc	0.0659	0.5562	0.0401	0.7205	0.1146	0.3054	0.3989	**0.0002**
PK	0.0273	0.8075	-0.0218	0.8458	0.3324	**0.0023**	0.2838	**0.0098**
MK-4	0.2725	**0.0132**	0.1882	0.0905	0.2022	0.0685	0.2059	0.0634
MK-7	-0.0463	0.6795	-0.0039	0.9725	0.1025	0.3596	0.1338	0.2307
Fat	0.0840	0.5027	-0.1460	0.2422	0.4596	**0.0001**	0.1565	0.2095

[1] Values listed in bold face showed significant correlation.

Discussion

For the determination of vitamin D, HPLC with ultraviolet detection [16], CPBA [17], RIA [18] and EIA [19] are used widely. However, there are several problems in sensitivity, specificity and accuracy with determination of vitamin D compounds in breast milk because concentrations of them are markedly low. In addition, CPBA, RIA and EIA are unable to distinguish each form of vitamin D. Our proposed method, including two extraction methods and sensitive LC-MS/MS detection using stable isotope-labeled internal standards, makes it possible to determine the principal fat-soluble vitamins in breast milk which contain more interfering compounds compared to plasma or serum samples. D and 25(OH)D could be measured by LC-MS/MS after DMEQ-TAD derivatization with the equivalent of only 6 mL of breast milk. DMEQ-TAD, a reagent originally developed for fluorescence-labeling, is highly sensitive and stable for conjugated dienes [23]. Recently, DMEQ-TAD has been used for the derivatization of vitamin D metabolites to improve ionization efficiency of LC-MS/MS with APCI [22]. In this study, DMEQ-TAD derivatization enhanced the measurement sensitivity of D and 25(OH)D by about 40 times. In addition, other fat-soluble vitamins were also measured sensitively compared to the standard assay methods, HPLC with fluorescence detection for RE [24], tocopherol [25] and vitamin K derivatives [26] and HPLC with visible detection for β-carotene [27].

We applied this method to a nutrition survey for lactating mothers. The mean concentrations of vitamins D were low in breast milk as reported previously [7–10]. The concentrations of 25(OH)D in breast milk [25(OH)D_3, 0.081±0.037 ng/mL; 25(OH)D_2, 0.003±0.002 ng/mL] were markedly low compared to plasma concentrations of 25(OH)D, which is the most abundant circulating metabolite of vitamin D with a concentration of 20-50 ng/mL in normal subjects. In contrast, the concentrations of D in breast milk [D_3,

0.088±0.128 ng/mL; D_2, 0.078±0.156 ng/mL] were comparable to 25(OH)D_3 although plasma levels of D_3 and D_2 are normally lower than 25(OH)D (1–5 ng/mL). These are significant correlations between the concentrations of D_3 and D_2 or 25(OH)D_3 and 25(OH)D_2; however, correlations between D and 25(OH)D were not found. Fat concentrations also correlated significantly and positively with 25(OH)D_3. These results suggest that the rate of secretion of vitamin D compounds from plasma to breast milk may be different depending on their polarity or affinity for plasma protein. Moreover, age of lactating mothers also correlated significantly and positively with the concentration of D_3 in breast milk. There is a possibility that higher intake of foods containing vitamin D including fish in older mothers could linked to higher concentration of D_3 in breast milk.

The amount of biological activity contributed by each vitamin D metabolites was proposed by Reeve et al. [28] as follows: D=1; 25(OH)D=5; 1,25$(OH)_2$D=10; and 24,25$(OH)_2$D=5. Mean content of total vitamin D in breast milk could be calculated using mean concentration of D_3, D_2, 25(OH)D_3 and 25(OH)D_2 [D_3, 0.088 ng/mL; D_2, 0.078 ng/mL; 25(OH)D_3, 0.081 ng/mL; 25(OH)D_2, 0.003 ng/mL] and Reeve's conversion factor as approximately 0.6 ng/mL. Suzuki et al. [29] reported that the mean intake of breast milk per day in Japanese breast-fed infants aged 1–5 months old was approximately 780 mL. The average intake of vitamin D in breast-fed infants was estimated to be 0.47 μg/day, which is extremely lower than current Dietary reference Intakes (DRIs) in Japan [adequate intake (AI) of vitamin D for infants 0–5 months, 5 μg/day)]. Thus, supplementation of vitamin D for breast feeding mothers or breastfed infants could be beneficial in improving the vitamin D status of breast fed infants especially during the winter.

In this study, post-partum days not correlated significantly with the concentrations of vitamin D in breast milk. Generally, vitamins A and E are high in colostrum, and decreased

and stable in mature breast milk. Sakurai et al. [30] reported that the concentrations of RE, β-carotene and α-Toc in breast milk decreased as the duration of lactation increased; however, clear correlations between the concentration of D_3 and the stage of lactation were not observed. Kojima et al. [14] demonstrated that PK and MK-4 concentrations in breast milk were high in colostrum and decreased during the course of lactation. Taken together, these results suggest that the distributions of each fat-soluble vitamin in breast milk might vary according to the duration of lactation and the concentrations of vitamin D in breast milk were not influenced by the stage of lactation unlike vitamins A, E and K.

In conclusion, we show a quantification method for fat-soluble vitamins in breast milk by LC-MS/MS. The present method maximizes the sensitivity and selectivity of the latest generation of tandem mass spectrometry and derivatization technique for the measurement of representative compounds of fat-soluble vitamins in human breast milk. The assay includes vitamins with a wide range of polarity, and this method has the advantage of low sample volume requirement. This method can apply the measurement of fat-soluble vitamins in other biological samples such as plasma, and may be useful for nutritional epidemiology studies and the setting of Dietary Reference Intakes of fat-soluble vitamins.

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CAPTER 2

Measurement of serum level of 25-hydroxyvitamin D in Japanese adolescent

Introduction

The major roles of Vitamin D are regulation of calcium homeostasis and bone metabolism. Among the vitamin D metabolites, serum 25(OH)D concentration is the best indicator of vitamin D status. Long-term vitamin D insufficiency can cause secondary hyperparathyroidism, which adversely affects bone metabolism in the elderly [1]. Negative correlations between serum 25(OH)D and PTH concentrations in children and adolescents have been reported in Argentina [2], France [3] and other countries [4–7]. However, there is limited evidence regarding the associations between vitamin D status and serum PTH concentration in Japanese adolescents. Serum PTH concentration is known to be regulated not only by vitamin D status, but also by dietary calcium intake [8]. Furthermore, dietary calcium intake in Japanese, including adolescents, is lower than in Europeans and Americans [9, 10]. These information suggest that vitamin D status may be more important for regulating serum PTH concentrations in adolescents with low calcium intake, such as Japanese adolescents.

Differences in the importance of vitamin D for bone growth between boys and girls are expected to exist during puberty because of differences in patterns of bone growth. It is known that boys tend to gain greater bone mass and density at a greater speed after age 10 though boys and girls develop bone mass at the same rate before puberty. Girls have a shorter window of time to gain their peak bone mass. However, little is known about sex differences in the associations between vitamin D status and serum PTH concentration and bone metabolism in adolescents.

In this study, we measured serum level of 25(OH)D in a large group of Japanese adolescent consisting of Junior and Senior High School students using automated competitive chemiluminescence immunoassay (CLIA) [11]. We provided comparable data

on vitamin D status to allow the establishment of a reference values of serum 25(OH)D concentration or vitamin D intake in adolescents and examined sex differences in the association between serum 25(OH)D concentration and serum PTH concentration or vitamin D intake.

Materials and methods

Subjects.

We recruited the adolescents aged 12–18 years (n=1415) through an annual health examination at a Junior and Senior High School located in urban Tokyo. They were all selected from the same Junior and Senior High School. Subjects who had suffered from acute infection or who had chronic diseases such as diabetes mellitus, kidney disease, bone metabolic disease or inheritable metabolic diseases were excluded. Also subjects whose serum parameters could not be measured due to less amount of blood sample were also excluded. Finally, a total of 1,380 healthy adolescents from 1st grade of junior high school (1st JHS) (192 boys and 197 girls, aged 12–13 years), 1st grade of high school (1st HS) (247 boys and 279 girls, aged 15–16 years), and 3rd grade of high school (3rd HS) (223 boys and 242 girls, aged 17–18 years) were enrolled. Health status of the participants was defined based on health history, questionnaire, and serum biochemical parameters. The comprehensive study protocol, including nutritional evaluation, was reviewed by the ethics committee of Kagawa Nutrition University and comprehensive written informed consent was obtained from all participants.

Measurements.

Blood samples were obtained in May 2003 and 2004. Fasting blood samples were collected by venipuncture at school in the morning, centrifuged at 1,940 × g for 15 min at 4°C, and the supernatant was stored at −35°C until assayed. Serum 25(OH)D was determined using the LIAISON 25OH Vitamin D TOTAL assay (DiaSorin Inc, Stillwater, MN, USA), which is an automated CLIA [11]. The LIAISON 25OH Vitamin D TOTAL assay is a rapid automated method with first results available in 40 min, and a subsequent

throughput of 180 samples per hour. Circulating level of intact PTH was measured by CLIA (LIAISON® N-TACT® PTH II Assay). Height, weight, and body mass index were measured for all subjects, and they also completed a questionnaire on exercise history, diet, and lifestyle factors. Calcaneal skeletal status was evaluated by quantitative ultrasound (QUS) measurements at the heel, using the Achilles system A-1000 (GE-Lunar, Madison, WI, USA), which measures speed of sound (SOS) in m/s and broadband ultrasound attenuation (BUA) in dB/MHz. The Achilles software was also used to calculate a stiffness index, which is a combination of both BUA and SOS. Calcaneal stiffness Z-score were calculated using Japanese age-matched reference data provided by GE-Lunar (Madison, WI, USA) as follows;

Z-score = (calculate a stiffness – reference data) / SD

Vitamin D and calcium intake were assessed using a food-frequency questionnaire (FFQ) [12], based on the semi-quantified FFQ developed by the Drafting Committee of the Ministry of Health and Welfare for Health Index. The FFQ has been shown to be a useful tool for evaluating dietary calcium and vitamin D intakes (coefficients of variance of four repeated measurements of intakes throughout 1 year were 14.1% for calcium, 13.6% for vitamin D).

Statistical Analysis.

All statistical analyses were performed using statistical software JMP 7.0 J (SAS Institute Inc, Cary, NC, USA). Analysis of variance (ANOVA) was performed to determine the significance of differences in anthropometric parameters, serum 25(OH)D and intact PTH concentrations, and vitamin D and calcium intakes among school grades. Student's t-tests were used to compare parameters between the sexes.

No definite 25(OH)D threshold for defining vitamin D deficiency/insufficiency has yet been established, and the proposed reference value varies among studies [13–15]. The Institute of Medicine of the National Academies in US/Canada recently proposed 50 nmol/L (20 ng/mL) 25(OH)D as a reference value to define vitamin D sufficiency [16]. Based on these reports, we evaluated the frequencies of vitamin D deficiency/insufficiency using the following serum 25(OH)D concentrations: <12.5 nmol/L (<5 ng/mL), severe vitamin D deficiency; 12.5–<25 nmol/L (5–<10 ng/mL), vitamin D deficiency; 25–<50 nmol/L (10–<20 ng/mL), mild vitamin D deficiency; 50–<75 nmol/L (20–<30 ng/mL), vitamin D insufficiency; ≥75 nmol/L (≥30 ng/mL), vitamin D sufficiency.

Threshold calcium intake values were based on the recommended daily allowances (RDAs) according to the DRIs for Japanese 2010 [17] (boys: 1000 mg/day (12–14 years), 800 mg/day (15–18 years); girls: 800 mg/day (12–14 years), 650 mg/day (15–18 years)) (<RDA: L-Ca, ≥RDA: H-Ca). Differences in calcaneal stiffness Z-score among the four groups were evaluated by ANOVA and Tukey–Kramer's honest significant difference test.

Results

The subject characteristics.

The subject characteristics are summarized in Table 1.

Table 1 Subject characteristics[1,2]

Parameter	All	School grade			ANOVA (among age groups)
		1st JHS (12–13 years)	1st HS (15–16 years)	3rd HS (17–18 years)	
Boys					
n	662	192	247	223	
Body height (cm)	165.3±9.9***	153.8±7.6	169.2±5.9***	171.5±5.4***	<0.001
Body weight (kg)	56.7±12.0***	45.3±9.0	59.2±9.4***	64.7±8.8***	<0.001
BMI (kg/m^2)	20.6±3.0	19.0±2.8	20.6±2.7	22.0±2.8***	<0.001
25(OH)D (nmol/L)	60.8±18.3***	58.8±15.5*	61.3±18.5***	62±19.8***	0.179
Intact PTH (pg/mL)	39.0±17.1	44.7±18.1	40.1±17.8	32.6±12.8	<0.001
Achilles stiffness	99.2±18.4**	85.6±11.8***	100.8±16.1	109.3±18.3***	<0.001
Achilles Z-score	−0.01±0.16***	−0.05±0.13***	−0.04±0.15***	0.06±0.18*	<0.001
Vitamin D intake (μg/d)	10.0±2.7	9.9±2.9	10.1±2.5	9.9±2.7	0.808
Ca intake (mg/d)	554±289***	555±282	562±315***	553±288***	0.945
Exercise (%)	69.7***	73.9***	64.5***	71.9***	0.071
% of outdoor exercise	69	73	74	58	
Girls					
n	718	197	279	242	
Body height (cm)	156.9±5.7	153.5±5.4	158.2±5.3	158.1±5.3	<0.001
Body weight (kg)	50.6±7.5	45.8±7.0	52.2±7.4	52.6±6.3	<0.001
BMI (kg/m^2)	20.5±2.5	19.4±2.5	20.8±2.7	21.0±2.1	<0.001
25(OH)D (nmol/L)	52.8±17.0	55.5±15.0	53.0±16.8	50.3±18.5	0.005
Intact PTH (pg/mL)	38.0±15.4	43.4±17.3	38.8±14.9	32.1±11.8	<0.001
Achilles stiffness	96.4±15.2	91.4±13.1	98.5±16.8	98.1±14.0	<0.001
Achilles Z-score	0.02±0.16	0.002±0.14	0.03±0.17	0.03±0.15	0.108
Vitamin D intake (μg/d)	10.1±2.5	10.2±2.6	10.0±2.4	10.1±2.5	0.783
Ca intake (mg/d)	471±199	507±221	459±187	454±189	0.01
Exercise (%)	48.3	53.1	47.5	44.9	0.224
% of outdoor exercise	48	44	55	45	

[1] Values were calculated as means ± SD.

[2] Significant differences between boys and girls are shown with asterisks (*p<0.05, **p<0.001, ***p<0.001).

Serum 25(OH)D concentrations in boys and girls were 60.8±18.3 nmol/L (24.3±7.3 ng/mL) and 52.8±17.0 nmol/L (21.1±6.8 ng/mL), respectively. Serum 25(OH)D concentrations of girls were significantly lower than those of boys in all age groups.

Distribution of serum 25(OH)D concentration in adolescent.

Distribution of serum 25(OH)D concentration in adolescent boys and girls in Fig 1. Approximately 30% of boys and 48% of girls had less than 50 nmol/L of 25(OH)D concentration, and approximately 80% of boys and 90% of girls had less than 75 nmol/L of 25(OH)D concentration. Overall, obvious vitamin D deficiency, defined as serum 25(OH)D concentration <25 nmol/L, was observed in eight boys and 19 girls. There was no significant difference in serum 25(OH)D concentration among school grades in boys, but serum 25(OH)D levels decreased significantly with age in girls (Table 1). In 3rd HS of girls, serum 25(OH)D concentration was 50.3±18.5 nmol/L which was approximately 5 nmol/L lower

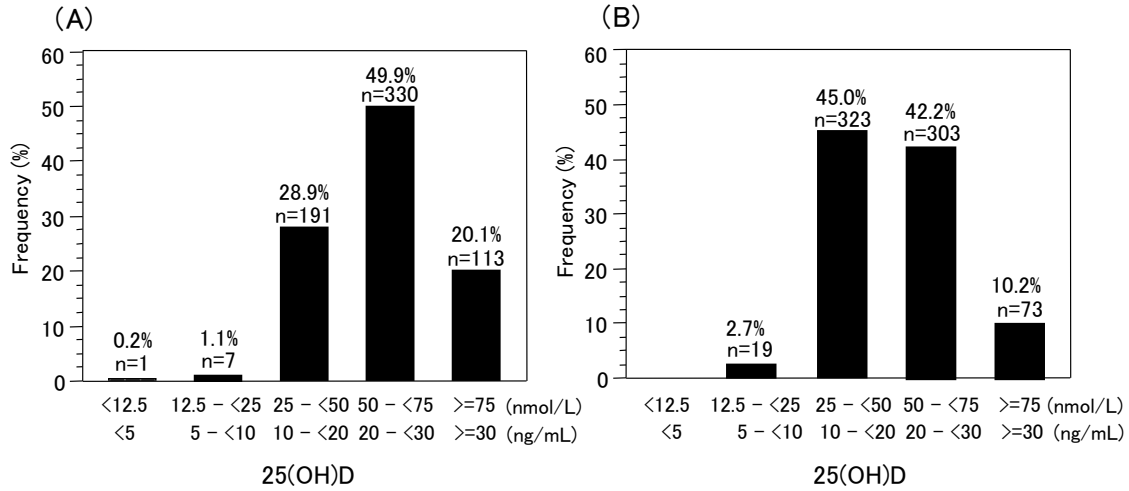

Fig. 1 Distribution of serum 25(OH)D concentration in adolescent boys (A) and girls (B). Vitamin D deficiency/insufficiency was defined using serum 25(OH)D concentration thresholds as follows; <12.5 nmol/L (<5 ng/mL), severe vitamin D deficiency; 12.5–<25 nmol/L (5–<10 ng/mL), vitamin D deficiency; 25–<50 nmol/L (10–<20 ng/mL), mild vitamin D deficiency; 50–<75 nmol/L (20–<30 ng/mL), vitamin D insufficiency; ≥75 nmol/L (≥30 ng/mL), vitamin D sufficiency.

than concentration of 1st JHS girls, and more than half of girls had less than 50 nmol/L of 25(OH)D concentration. Although vitamin D intake did not differ between boys and girls, serum 25(OH)D concentrations were lower in girls than in boys. On the other hand, exercise habit and the ratio of outdoor exercise were much higher in boys than in girls.

Regression analysis between serum 25(OH)D and intact PTH concentrations.

Intact PTH concentration in boys and girls were 39.0±17.1 pg/mL and 38.0±15.4 pg/mL, respectively (Table 1). Intact PTH concentration decreased significantly with age in both boys and girls, with no significant difference between the sexes. Decrements of intact PTH concentration from 1stJHS to 3rdHS in both boys and girls were approximately 10 pg/mL. Although calcaneal stiffness was higher in girls than in boys in 1st JHS (12–13 years), the high rate of bone growth in boys led to a reversal of this phenomenon in 3rd HS (17–18 years). In simple regression analysis, a negative correlation between serum 25(OH)D and PTH concentration was observed in boys (Fig. 2). In girls, however, significant correlation was observed only in 3rd HS. Calcium intake among high school students was higher in boys than in girls (Table 1).

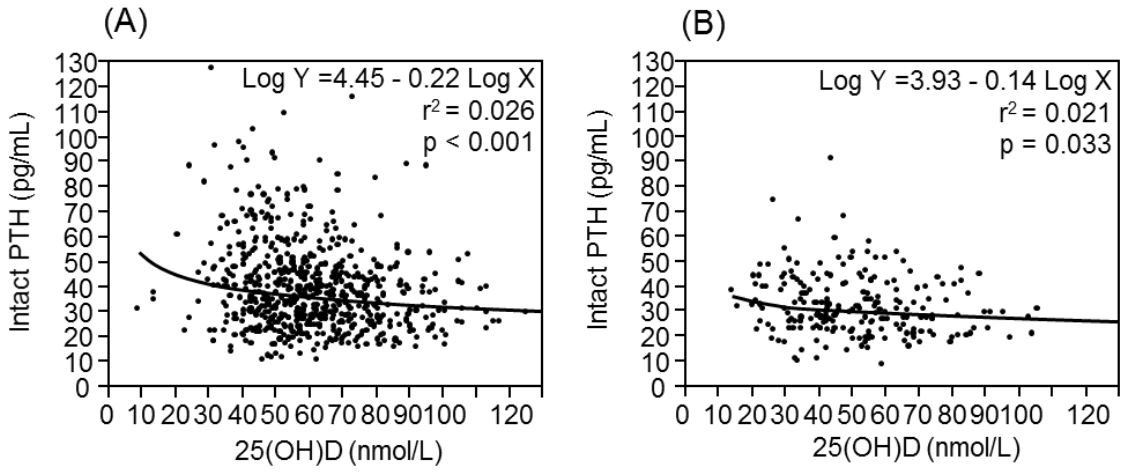

Fig. 2 Simple regression analysis between serum 25(OH)D and intact PTH concentrations in boys (A) and girls in 3rd HS (B).

Regression analysis between serum 25(OH)D and calcaneal stiffness.

Negative correlations between serum 25(OH)D and calcaneal stiffness in simple regression analysis were observed in both boys ($p=0.029$, $r^2=0.007$) and girls ($p<0.001$, $r^2=0.049$) (Fig. 3).

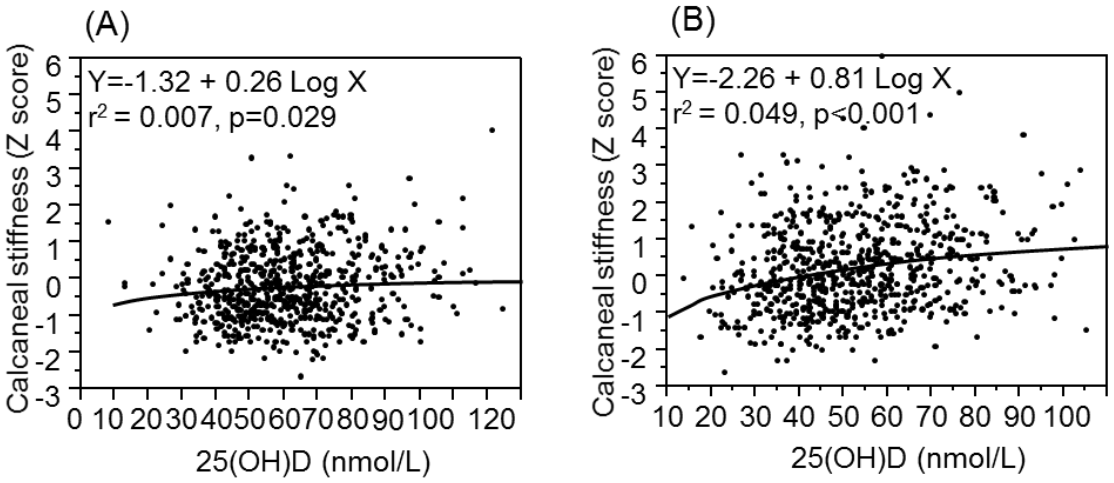

Fig. 3 Simple regression analysis between serum 25(OH)D and calcaneal stiffness Z score in boys (A) and girls (B).

Associations of vitamin D status and calcium intake with calcaneal stiffness Z-score.

Fig. 4 shows the associations of vitamin D status and calcium intake with calcaneal stiffness Z-score. The Z-scores in the H-25(OH)D groups were significantly higher than in the L-25(OH)D groups for boys(Fig. 4A) and girls (Fig. 4B). The difference between L-25(OH)D and H-25(OH)D groups was more significant in girls than in boys. Moreover, subgroup analysis identified significant and stronger associations of both vitamin D status and calcium intake in girls compared with boys (Fig.4C, D). These results suggest that calcaneal stiffness might be more susceptible to 25(OH)D concentration and calcium intake in girls than in boys. Also, Fig.4 indicates that vitamin D status has more impact on bone than Ca intake in both sexes.

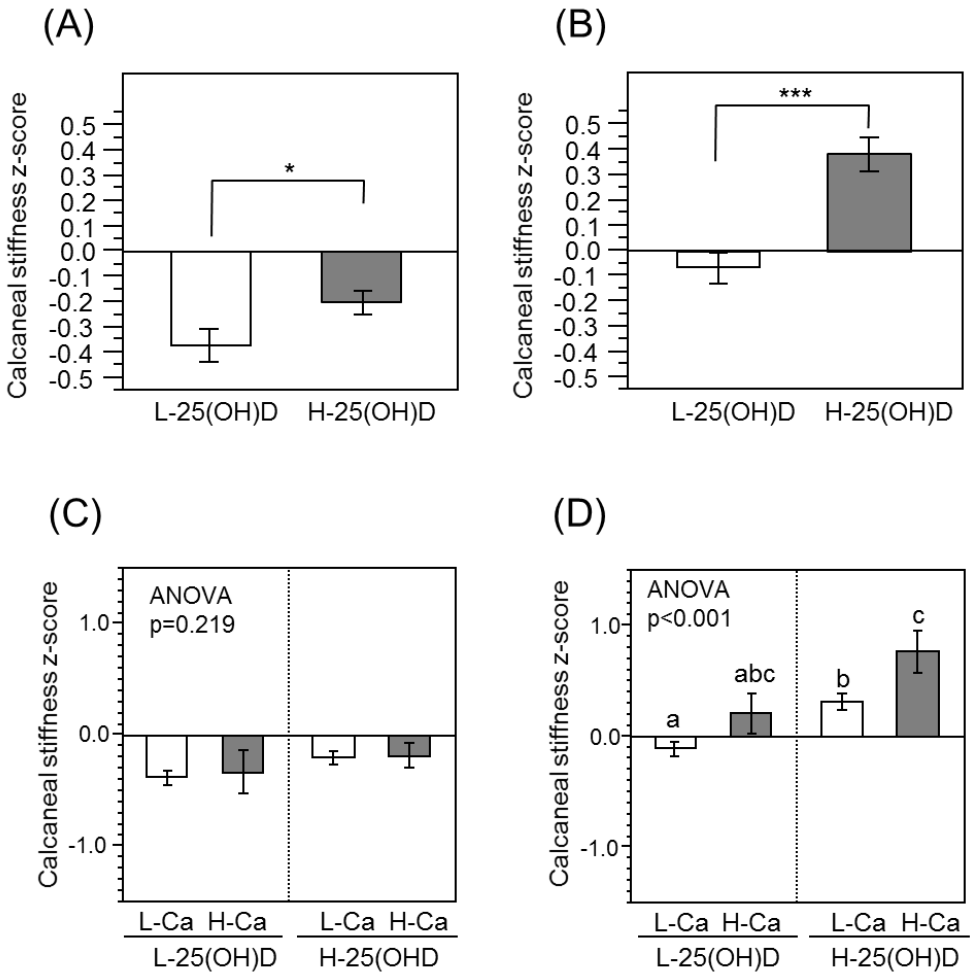

Fig. 4 Association between vitamin D status and calcium intake and calcaneal stiffness in boys (A and C) and girls (B and D). Subjects were divided according to serum 25(OH)D concentration and calcium intake. L-25(OH)D and H-25(OH)D; –<50 nmol/L and –≧50 nmol/L serum 25(OH)D concentration, respectively. L-Ca and H-Ca; –<800 mg/day and –≧800 mg/day calcium intake, respectively. A and B, L-25(OH)D and H-25(OH)D groups were compared using Student's t-test. *p<0.05, ***p<0.001; C and D, Difference in calcaneal stiffness z-score among the four groups were evaluated by ANOVA and Tukey-Kramer's honest significant difference test. Significant differences are between the groups which are not connected by the same letter (a, b or c). Values given are means and standard errors (SE).

Discussion

We assessed vitamin D status in Japanese adolescents to establish a reference values of serum 25(OH)D concentration. In this study, the mean serum 25(OH)D concentrations in boys and girls were 60.8±18.3 nmol/L (24.3±7.3 ng/mL) and 52.8±17.0 nmol/L (21.1±6.8 ng/mL), respectively. Serum 25(OH)D concentrations in Japanese adolescents were similar to those reported in the US and Europe [3, 4, 8, 18–22], and higher than those in China and India [23–25]. In this study, the serum 25(OH)D concentration in girls was significantly lower than in boys, and decreased significantly with age. However, González-Gross et al. reported that the 25(OH)D concentration was higher in girls than in boys and increased with age [22], while other studies showed a significant reduction in serum 25(OH)D concentration according to increasing age in adolescents [15], or lack of an association between 25(OH)D concentration and age [20]. These inconsistent results in adolescents suggest that region-specific lifestyles may be an important factor influencing 25(OH)D concentration during adolescence. The present study found that serum 25(OH)D concentration was lower in girls than in boys, although vitamin D intake did not differ between boys and girls. One possible explanation for this may be the higher percentage of boys taking exercise, and the higher ratio of outdoor exercise compared with girls.

A negative correlation between serum 25(OH)D and PTH concentration was observed in Japanese adolescents, in accordance with other studies [3–7]. A negative correlation between serum 25(OH)D and PTH concentration was observed in boys in all grades tested but only girls in 3rd HS. PTH concentration was more susceptible to serum 25(OH)D concentration in boys than in girls. These results suggest that serum PTH concentration is thought to be a useful marker of vitamin D insufficiency in adolescent Japanese boys aged 12–18 years. In girls, calcium intake had a greater association than serum 25(OH)D

concentration on serum PTH. One possibility is that an extremely low calcium intake (471 ± 199 mg/day) in Japanese adolescents may affect the relationship between PTH and 25(OH)D concentrations. The calcium intake of Japanese girls was approximately one third lower than that of Finnish girls [26]. These results suggest that serum PTH concentration may not be a useful marker of vitamin D insufficiency in girls aged 12–16 years who have a low calcium intake, such as Japanese adolescents.

The average vitamin D intake in Japanese adolescents was approximately 10 μg/day. This is two to three times higher than the AI according to the DRIs for the Japanese population (AI: 3.5 μg/day for 12–14-year-olds, 4.5 μg/day for 15–17-year-olds, 5.5 μg/day for 18–29-year-olds) [27]. However, approximately 30% of boys and 48% of girls had a blood concentration less than 50 nmol/L of 25(OH)D. Exposure to sunlight is known as the most important factor affecting serum 25(OH)D concentration. Therefore, the difference in sunlight exposure among subjects would influence serum 25(OH)D concentration. Taken together, higher vitamin D intake or much more sun exposure are needed to improve the status of vitamin D deficiency in Japanese adolescents.

The RDA of calcium according to the Dietary Reference Intakes for Japanese 2015 [17] is 1,000 mg/day for 12–14-year-olds and 800 mg/day for 15–18-year-olds in boys, and 800 mg/day for 12–14-year-olds and 650 mg/day for 15–18-year-olds in girls. Thus, the calcium intake of 450–550 mg/day in Japanese adolescents of both sexes was regarded as very low. Improvement of these low calcium status, especially in girls, should be importance for bone health in Japan.

Serum 25(OH)D concentration was significantly positively associated with calcaneal stiffness in adolescents. In contrast to the relationship between serum 25(OH)D and PTH concentration, serum 25(OH)D concentration was significantly associated with calcaneal

stiffness in girls of all age groups. The present study also suggested that both vitamin D status and calcium intake would have greater associations with calcaneal stiffness in adolescent girls. The reason why the association between 25(OH)D concentration and calcaneal stiffness was weaker in boys is unclear. After the growth spurt, bone mineral content increments were much higher in boys than in girls [28]. We also observed a much higher increment in calcaneal stiffness in boys. From body height, it could be assumed that ages 12-13 of boys are still in the early stages of puberty while the most of the girls at this age are near menarche. Also it could be assumed that girls reach final height by age 15, but boys don't reach until age 17. Sex differences in these bone growth may therefore be one reason why calcaneal stiffness was hardly affected by 25(OH)D concentration in adolescent boys.

To the best of our knowledge, the present study represents the first evaluation of vitamin D status in Japanese adolescents and could thus provide comparable data for establishing reference values of serum 25(OH)D concentration and vitamin D intake. This study is also the first to report a sex difference in the relationships between vitamin D status and PTH concentration and calcaneal stiffness in adolescents. However, the study was a cross-sectional study and subjects were recruited from only an urban area in the eastern part of Japan. Further studies involving more subjects of all age groups, from rural as well as urban areas in different parts of the country, are needed to verify the results. Additionally, concentrations of sex hormones and growth hormone and pubertal status would be associated with the sex difference in the relationships between vitamin D status and PTH concentration and calcaneal stiffness. Thus, comprehensive analysis including these hormones and pubertal status are also needed in the future.

Despite its limitations, the present study was able to conclude that vitamin D deficiency is common in Japanese adolescents. Vitamin D supplementation and sun exposure would be effective in improving vitamin D status in Japanese adolescent. We also confirmed that serum PTH concentration is a useful biomarker of vitamin D deficiency in Japanese adolescents, except in girls aged 12–16 years with low calcium intake. Moreover, the results of present study suggest that vitamin D status has a greater association with calcaneal stiffness in girls than in boys.

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PART 2

Identification of novel metabolites of vitamin D

CAPTER 1

Identification of C-3 epimers of native vitamin D

Introduction

D_3 is metabolized to 25(OH)D_3 in the liver and subsequently to the active form, 1α,25(OH)$_2D_3$, or the inactive form, 24,25(OH)$_2D_3$, in the kidney. After the expression of biological activities, 25(OH)D_3 and 1α,25(OH)$_2D_3$ are further metabolized *via* C-24 [1–3] or C-23/26 [4–7] oxidation pathways by type of cytochorome P450 (CYP) 24-hydroxylase, CYP24A1 [8, 9]. The C-24 oxidation pathway, initiated by C-24 hydroxylation, yields C-24 oxo compounds and ultimately results in the formation of side-chain cleavage products including calcitroic acid [2, 3]. The C-23 oxidation pathway, initiated by C-23 hydroxylation, results in the formation of metabolites with a lactone-ring in the side-chain including 1α,25(OH)$_2D_3$-26,23-lactone [4–7]. In addition to these traditional metabolic pathways, a novel C-3 epimerization pathway of 1α,25(OH)$_2D_3$ was identified [10–13]. The C-3 epimerization pathway leads to the conversion of the configuration of the hydroxyl group at C-3 of the A-ring and produces 3-epi-1α,25(OH)$_2D_3$ from 1α,25(OH)$_2D_3$. In view of this modification at the A-ring, the C-3 epimerization pathway is quite different from side-chain oxidation pathways.

The C-3 epimerization of 1α,25(OH)$_2D_3$ was observed in human colon carcinoma-derived Caco-2 cells [10], bovine parathyroid cells [11], rat osteosarcoma-derived UMR-106 and Ros17/2.8 cells [12] and various other cultured cell lines [13]. It was considered that the C-3 epimerization pathway is cell differentiation-related in Caco-2 cells, because 3-epi-1α,25(OH)$_2D_3$ was only observed in confluent, quiescent Caco-2 cells, not proliferating Caco-2 cells [10]. 3-Epi-1α,25(OH)$_2D_3$ was also isolated as a circulating metabolite of 1α,25(OH)$_2D_3$ in rats treated with pharmacological doses of 1α,25(OH)$_2D_3$ [14]. In addition, synthetic analogs of 1α,25(OH)$_2D_3$, e.g. 1α,25(OH)$_2$-16-ene-23-yne-D_3 [15] and 20-epi-1α,25(OH)$_2D_3$ [16], have been reported to be metabolized to their respective C-3 epimers.

It has been demonstrated that 3-epi-24,25$(OH)_2D_3$-24-glucuronide existed in the bile of rats administered pharmacological doses of 24,25$(OH)_2D_3$ [17]. 3-Epi-24,25$(OH)_2D_3$ was also identified in cell culture media [18] and rat plasma [19]. However, it is unclear whether 25$(OH)D_3$ would be metabolized to its C-3 epimer, 3-epi-25$(OH)D_3$. 25$(OH)D_3$ is the most abundant circulating metabolite of vitamin D_3 with a concentration of approximately 20 to 50 ng/mL under normal conditions [20]. 25$(OH)D_3$ is the immediate precursor of the active form, 1α,25$(OH)_2D_3$, and the inactive form, 24,25$(OH)_2D_3$. Thus, metabolism of 25$(OH)D_3$ is thought to be important for regulation of the biological activity of D_3.

In this study, we demonstrated the metabolism of 25$(OH)D_3$ in rat osteosarcoma UMR-106 cells and identified 3-epi-25$(OH)D_3$ using ^{1}H-NMR spectroscopy and LC-MS techniques. 3-Epi-25$(OH)D_3$ was also detected in various cell models cultured with 25$(OH)D_3$ *in vitro* and in the serum of rats administered pharmacological doses of 25$(OH)D_3$ *in vivo*. We also demonstrated that 3-epi-25$(OH)D_3$ was further metabolized by 1α-hydroxylase, CYP27B1 and 24-hydroxylase, CYP24A1.

Materials and methods

Materials.

3-epi-25(OH)D_3, 3-epi-1α,25$(OH)_2D_3$ and 3-epi-24,25$(OH)_2D_3$ were synthesized by Hatakeyama et al. of Nagasaki University. 1α,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ were obtained from Solvay Pharmaceuticals B.V. (Veenendaal, The Netherlands). 1α,24,25-trihydroxyvitamin D_3 [1α,24,25$(OH)_3D_3$] was kindly provided by Kureha Chemical Industry Co., Ltd. [26,27-methyl-^{3}H]-1α,25$(OH)_2D_3$ (179 Ci/mmol) and [23,24(n)-^{3}H]-25(OH)D_3 (82 Ci/mmol) were purchased from Amersham Biosciences (changed name to GE Healthcare). Deuterized chloroform ($CDCl_3$, 99.8 %, NMR analytical grade) was purchased from EURISO-TOP (Gif-Sur-Yvette, France).

Cell Culture.

A rat ostesarcoma cell line (UMR-106), human osteosarcoma cell line (MG-63), human colon adenocarcinoma cell line (Caco-2), porcine kidney cell line (LLC-PK_1) and human hepatoblastoma cell line (HepG2) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). UMR-106 and MG-63 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal calf serum (FCS). Caco-2 cells were maintained in Minimum Essential Medium (MEM) containing 10 % FCS and 1% non-essential amino acids. LLC-PK_1 cells were maintained in Medium 199 containing 5 % FCS. HepG2 cells were maintained in MEM containing 10 % FCS, 1% non-essential amino acids and 1% sodium pyruvate. All culture media contained penicillin (100 IU/ml) and streptomycin (100 μg/mL). Cells were cultured at 37 ºC in a humidified atmosphere of CO_2 in air with a change of medium every three days. In the experiments

described below, vitamin D_3 compounds were added to the culture medium in an ethanolic solution, the final concentration in the medium never exceeding 0.1 % (v/v).

Generation of Vitamin D Metabolites in Cultured Cells.

UMR-106, MG-63 and LLC-PK_1 cells (2×10^6) and Caco-2 and HepG2 cells (4×10^6) were seeded in 150-mm culture dishes and cultured for 4 days to late log phase. The medium was removed and cells were washed with phosphate-buffered saline without Ca, Mg [PBS (−)] and then in medium containing 1 % bovine serum albumin (BSA) in the presence of 10 μM of 25(OH)D_3, 1α,25$(OH)_2D_3$, 24,25$(OH)_2D_3$, 3-epi-25(OH)D_3 or 3-epi-1α,25$(OH)_2D_3$ for 48 h at 37ºC. For measurements of vitamin D_3 metabolites, three 150-mm culture dishes were used for each culture. For time-course experiments, the cells were incubated with 5 μM of 25(OH)D_3, 3-epi-25(OH)D_3 or 1 μM of 1α,25$(OH)_2D_3$, 3-epi-1α,25$(OH)_2D_3$ for a period of time ranging from 1 to 48 h.

Purification of Metabolites.

Lipid extraction was performed according to the method of Bligh and Dyer [21] as modified by Makin et al. [2]. Lipid was extracted from cells and medium with methanol and dichloromethane. The organic layer, containing unchanged substrate and lipid-soluble metabolites, was evaporated under nitrogen gas to dryness, and the residue was redissolved in hexane/2-propanol/methanol (HIM), (88:10:2, v/v/v). HPLC was carried out using a model 600 pump and a model 996 photodiode array detector (Waters Associates, Milford, MA). Elution was performed on a Zorbax SIL column (4.6 × 250 mm, Agilent Technologies) using HIM (88:10:2), at a flow rate of 1.0 mL/min (first HPLC system). Metabolites were identified based on UV characteristics of the vitamin D *cis*-triene system

(λmax=265 nm, λmin=228 nm). The same metabolites were also separated on a Sumichiral OA-2000 column (4.6 × 250 mm, Sumika Chemical Analysis Service, Ltd., Osaka, Japan) using 2-propanol/hexane (96.5/3.5 or 94.5/5.5, v/v) or a Zorbax CN column (4.6 × 250 mm, Agilent Technologies) using HIM(88:10:2), at a flow rate of 1.0 mL/min (second HPLC system). Concentrations of stock solutions of D_3-related compounds were determined spectrophotometrically using a molar extinction coefficient, ε265=18,200.

^{1}H-NMR and LC-MS Analyses.

The 500-MHz ^{1}H-nuclear magnetic resonance (NMR) spectra of the isolated metabolites were measured on a Varian VXR-500 (^{1}H: 499.9 MHz). Purified metabolites (3 μg) were dissolved in 40 μL of $CDCl_3$ with a very small portion of $CHCl_3$ (7.24 ppm, used as an internal standard for ^{1}H-NMR spectroscopy) and transferred into a nano probe. 2 Dimensional Correlation Spectroscopy (2D COSY) and 2 dimensional Nuclear Overhauser Effect Correlated Spectroscopy (2D NOESY) spectra were obtained as described previously [18]. Liquid chromatography mass spectrometry (LC-MS) analysis was carried out with a QUATTROII (Waters Micromass, Manchester, U.K.) equipped with an electrospray ionization (ESI) source in the positive ion mode. An HPLC system consisting of a MAGIC2002 Micro-LC full system (Michrom Bio Resources, Inc, CA) and a Develosil ODS-HG-5 column (2.0 × 150 mm, NOMURA CHEMICAL, Tokyo, Japan) was used. As a mobile phase, methanol/10 mM ammonium acetate (50/50, v/v: A) and methanol/10 mM ammonium acetate (98/2, v/v: B) were used and a linear gradient elution was run from an A/B ratio of 65/35 to 20/80, at a flow rate of 0.2 mL/min. The column temperature was maintained at 40 ºC. Mass spectra were obtained by averaging each peak and subtracting the background.

In Vivo Metabolism of 25(OH)D_3 in Rats

Male Wistar rats (Japan SLC, Hamamatsu, Japan), weighing approximately 300 g, were used in experiments, following adaptation to laboratory conditions for at least 5 days. Three rats were given a bolus dose of 500 μg of 25(OH)D_3 intravenously. At 6 h after dosing, each rat was sacrificed and the blood was collected, heparinized and immediately centrifuged at 1,940 × g. About 5 mL of serum was obtained from each rat. Lipid extraction of serum and purification of 25(OH)D_3 metabolites were performed as described in the "Purification of Metabolites" section.

Metabolism of 3-Epi-25(OH)D_3 in Cultures of Recombinant E. Coli Cells Expressing CYP27B1 and CYP24A1.

Co-expression plasmids for human CYP27B1, bovine adrenodoxin (ADX) and nicotinamide adenine dinucleotide phosphate, reduced form (NADPH)-adorenodoxin reductase (ADR) (pKSNdl-CYP27B1) were constructed as described previously [22]. Co-expression plasmids for CYP24A1, ADX and ADR (pKSNdl-CYP24A1) were reported previously by Sakaki et al. [23]. Recombinant *E. Coli* cells transfected with the above plasmids (JM109/pKSNdl-CYP27B1 or JM109/pKSNdl-CYP24A1) were grown in TB medium [24] containing 50 μg/ml of ampicillin at 26 ºC. The induction of transcription was initiated by addition of isopropyl thio-β-D-galactoside at a final concentration of 1 μM when the cell density (OD660) reached 0.5. Then, 50 μM of 25(OH)D_3 or 3-epi-25(OH)D_3 was added to the culture and the cells were incubated for 48 h. Lipid extraction and purification of metabolites were performed as described in the "Purification of Metabolites" section. Recombinant *E. Coli* cells transfected with pKSNdl derived from pKK233-3 (JM109/pKSNdl) were used for control experiments.

VDR and DBP Binding Assay.

Displacement of [^{3}H]-1α,25(OH)$_2$D$_3$ from calf-thymus cytosol receptors (Yamasa Co. Ltd., Chiba, Japan) by 1α,25(OH)$_2$D$_3$, 25(OH)D$_3$, 24,25(OH)$_2$D$_3$ and their C-3 epimers was determined as described previously [18, 25]. Solutions containing 500 μL of the calf-thymus cytosol receptor prepared with phosphate buffer (0.3 M KCl, 0.05 M K_2HPO_4, 0.05M KH_2PO_4, pH 7.4) were mixed with increasing amounts of 1α,25(OH)$_2$D$_3$ (0.0078–64 pg/tube), 3-epi-1α,25(OH)$_2$D$_3$, 25(OH)D$_3$, 3-epi-25(OH)D$_3$, 24,25(OH)$_2$D$_3$ and 3-epi-24,25(OH)$_2$D$_3$ (0.0078–16384 pg/tube) in 20 μL ethanol, and the samples were incubated at 20 ºC for 1 hr. Next, 34 fmol [^{3}H]- 1α,25(OH)$_2$D$_3$ in 25 μL ethanol was added, and the samples were incubated at 20 ºC for 1 hr. The addition of 200 μL of dextran/charcoal (0.05% dextran T-150/0.5% Norit A Charcoal Decolorizing Neutral) in freshly prepared phosphate buffer (0.05 M Na_2HPO_4, 0.05M NaH_2PO_4, pH 7.4) was used to separate the bound and free forms of [^{3}H]-1α,25(OH)$_2$D$_3$. The assay tubes were incubated on ice for 10 min and centrifuged at 1,940 × g for 10 min at 4 ºC. Each supernatant was collected 500 μL and transferred into a scintillation vial to measure radioactivity. Competitive displacement of [^{3}H]- 25(OH)D$_3$ from vitamin D-deficient rat serum DBP by 1α,25(OH)$_2$D$_3$, 25(OH)D$_3$, 24,25(OH)$_2$D$_3$ and their C-3 epimers was determined under equilibrium ligand-binding conditions [25]. A total of 82 fmol [^{3}H]-25(OH)D$_3$ in 50 μL ethanol was mixed with increasing amounts of 25(OH)D$_3$, 24,25(OH)$_2$D$_3$ and their C-3 epimers (0.003125-16 ng/tube), 1α,25(OH)$_2$D$_3$ and 3-epi-1α,25(OH)$_2$D$_3$ (0.24-819.2 ng/tube) in 100 μL ethanol. Next, 1 mL of vitamin D- deficient rat serum diluted 1:70,000 with freshly prepared barbital acetate buffer (3.5 mM acetic acid, 3.5 mM sodium barbiturate, 0.13 M NaCl, 0.1% ovalbumin, pH 8.6) was added, and the samples were incubated on ice for 1 hr. To separate

the bound and free forms of [^{3}H]-25(OH)D_3 , 500 μL of dextran/charcoal (0.025% dextran T-150/0.25% Norit A Charcoal Decolorizing Neutral) in freshly prepared barbital acetate buffer was used. The assay tubes were vortexed and centrifuged at 1,940 × g for 10 min at 4 ºC. Following centrifugation, 1.0 ml of each supernatant was collected and transferred into a scintillation vial to measure radioactivity. The binding affinity of 1α,25$(OH)_2D_3$, 25(OH)D_3, 24,25$(OH)_2D_3$ and their C-3 epimers for the VDR was tested using a calf thymus 1α,25$(OH)_2D_3$ receptor assay (Yamasa Co., Chiba, Japan). The receptor was incubated at 20 ºC for 1 h with increasing concentrations of a D_3 compound and then 15,000 dpm of [^{3}H]-1α,25$(OH)_2D_3$ was added and incubated at 20 ºC for 1 h as described previously [18, 25]. The binding affinity of 1α,25$(OH)_2D_3$, 25(OH)D_3, 24,25$(OH)_2D_3$ and their C-3 epimers for the vitamin D binding protein (DBP) was tested using plasma from vitamin D-deficient rats [26]. The displacement of [^{3}H]-25(OH)D_3 from vitamin D-deficient rat plasma diluted (1: 70,000) 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 [18, 25].

Transfection and Luciferase Activity Assay

MG-63 was maintained in DMEM supplemented with penicillin (100 IU/mL), streptomycin (100 μg/mL) and 10 % dextran-coated charcoal-treated FCS. Cells (2×10^5) were suspended in 2 mL of the medium and transfected with 0.5 μg of luciferase reporter plasmid (pGVB2 vector, Toyo Ink Co., Ltd., Tokyo, Japan) containing a human osteocalcin gene promoter (−848/+10) including a vitamin D-responsive element (VDRE) [27] or a rat CYP24 gene promoter (−291/+9) including two VDREs [28] and 0.25 μg of pRL-CMV vector (pGVB2 vector, Toyo Ink Co., Ltd.) as an internal control using Tfx-50 reagent

(Promega Corp. Madison, WI). The cells were incubated with 1α,25(OH)$_2$D$_3$ (10^{-11}–10^{-7} M) or the other D$_3$ compounds (10^{-9}–10^{-6} M) for 48 h. The luciferase activities of the cell lysates were measured with a luciferase assay system (Toyo Ink Co., Ltd.), according to the manufacturer's instructions. Transactivation measured as luciferase activity was standardized with the luciferase activity of the same cells determined with the Sea Pansy luciferase assay system as a control (Toyo Ink Co., Ltd.) [29].

Anti-Proliferative Activity Assay.

The human promyelocytic leukemia cells (HL-60) were kindly provided by Dr. M. Inaba, Osaka City University Medical School, Japan. Cells were cultured at 37 ºC in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% FCS and 60 mg/L of kanamycin. For flow cytometry, HL-60 cells (10^5 cells/well) were placed in 24-well tissue culture plates and cultured for 3 days with 1α,25(OH)$_2$D$_3$ (10^{-11}–10^{-7} M) or the other D$_3$ compounds (10^{-9}–10^{-6} M). Each group of cells was washed with PBS (−) and resuspended in PBS (−) containing 0.2% Triton-X and 100 μg of RNase, and incubated at 37 ºC for 1 h. Cells were washed with PBS (−) and incubated with 0.5 mL of deoxyribonucleic acid (DNA)-staining solution containing propidium iodide (50 μg/mL) at 4 ºC for 20 min. The cells were analyzed with a flow cytometer equipped with an argon laser (488 nm, Becton Dickinson FACScan^{TM}) and the cell cycle distribution was analyzed using ModiFiT LT (Verity).

Analysis of Cell Surface Antigen Expression.

For the analysis of cell surface expression of CD-11b antigen, HL-60 cells (10^5 cells/well) were cultured for 3 days with 1α,25(OH)$_2$D$_3$ (10^{-11}–10^{-7} M) or the other D$_3$

compounds (10^{-9}–10^{-6} M) under the same conditions as for flow cytometry. Each group of cells was washed with PBS (−) and the cells (2×10^5 cells) were resuspended in 100 μL of diluent solution containing 1% BSA and 1% sodium azide. Then the cells were incubated with 10 μL of human monoclonal FITC-conjugated CD11b antibody (Sigma-Aldrich, St. Louis, MO, 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 FACScan™ at an 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.

Statistics

Values were calculated as mean ±SE. Significance levels were determined by Student's t-test.

Results

Metabolism of 25(OH)D_3 in UMR-106 Cells.

After incubation of 10 μM of 25(OH)D_3 with UMR-106 cells for 48 h, lipid extracts from the media along with the cells were subjected to a first HPLC using a Zorbax SIL column (Fig.1A). The major peak, peak 2 corresponding to the authentic standards of 25(OH)D_3 [retention time (R.T.) 5.67 min] and 3-epi-25(OH)D_3 (R.T. 5.68 min), was collected in a single fraction eluting between 5 and 7 min (Fraction X). Fraction X was then subjected to a second HPLC using a Sumichiral OA-2000 column for the separation of 3-epi-25(OH)D_3 (R.T. 16.92 min) from 25(OH)D_3 (R.T. 18.14 min) (Fig. 1B). The middle peak (peak b, R.T. 16.95 min), which possesses the characteristic UV chromophore of vitamin D_3 (λmin 228 nm, λmax 265 nm), exhibited the same retention time as authentic 3-epi-25(OH)D_3 but a different retention time to 25(OH)D_3. The metabolite corresponding to peak b was not formed when 25(OH)D_3 was incubated with the medium alone. It is clear from the HPLC chromatograms that peak b is the major metabolite of 25(OH)D_3 produced in UMR-106 cells. Peak b was collected in sufficient quantity to permit identification by ^{1}H-NMR and LC-MS analyses. Peak a (R.T. 16.30 min), which possesses the characteristic UV chromophore of previtamin D_3 (λmin 228 nm, λmax 260 nm), was also observed in a control incubation with no cells, only medium. Peak 4 in the first HPLC system corresponding to authentic standards of 24,25$(OH)_2D_3$ (R.T. 8.27 min) and 3-epi-24,25$(OH)_2D_3$ (R.T. 8.16 min) was collected (Fraction Y) and purified twice as described previously [20]. 24,25$(OH)_2D_3$ was contained in this fraction, however 3-epi-24,25$(OH)_2D_3$ was not detected (data not shown).

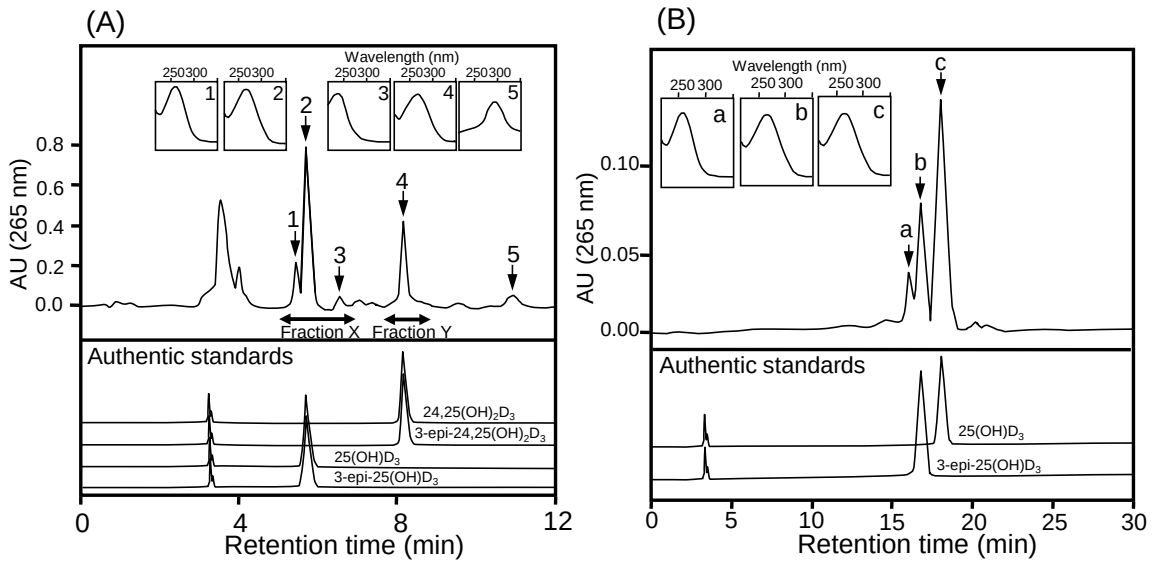

Fig.1 Metabolism of 25(OH)D_3 in cultured rat osteosarcoma UMR 106 cells. (A)Upper panel, The first HPLC profile and UV spectra of the lipid extracts from UMR-106 cells along with the medium incubated with 10 μM of 25(OH)D_3 for 48 h. Lower panel, The HPLC profiles of authentic standards of 25(OH)D_3, 3-epi-25(OH)D_3, 24,25$(OH)_2D_3$ and 3-epi-24,25$(OH)_2D_3$. The first HPLC analysis was performed using a Zorbax-SIL column (4.6 x 250 mm) eluted with hexane/2-propanol/methanol (HIM 88/10/2, v/v/v) at a flow rate of 1.0 mL/min. (B)Upper panel, The second HPLC profile and UV spectra of Fraction X obtained from the first HPLC. Lower panel, The HPLC profiles of authentic standards of 25(OH)D_3 and 3-epi-25(OH)D_3. The second HPLC was performed using a Sumichiral OA-2000 column (4.6 x 250 mm) eluted with 2-propanol/hexane (96.5/3.5, v/v) at a flow rate of 1.0 mL/min.

Identification of 3-Epi-25(OH)D_3 by ^{1}H-NMR and LC-MS Analyses.

The ^{1}H chemical shifts assigned based on 1D and 2D COSY, NOESY spectra of synthetic standards and the 25(OH)D_3 metabolite (Peak b) are summarized in Table 1. In the ^{1}H-NMR spectrum, the most pronounced difference between 25(OH)D_3 and 3-epi-25(OH)D_3 was the chemical shift of H-3 [25(OH)D_3: 3.93 ppm, 3-epi-25(OH)D_3: 3.86 ppm]. The chemical shift of H-3 of Peak b (3.86 ppm) was observed at the same position as 3-epi-25(OH)D_3 upfield in comparison with 25(OH)D_3. Such an upfield shift appears to be responsible for the C-3 epimerization in the A-ring of 25(OH)D_3. The resonances from all the protons except H-3 were proof of an unchanged side-chain as well as the *cis*-triene system. In the LC-MS spectra of authentic 25(OH)D_3 and 3-epi-25(OH)D_3, $[M+H]^+$ and

$[M+NH_4]^+$ were observed at m/z 401.8 and 418.8 (Table 1). In the spectrum of Peak b, $[M+H]^+$ and $[M+NH_4]^+$ were also observed at m/z 401.8 and 418.8. Thus Peak b was considered to be a diastereomer or a geometric isomer of $25(OH)D_3$. From the results of HPLC, ^{1}H-NMR and LC-MS analyses, Peak b could be assigned as 3-epi-$25(OH)D_3$, a diastereomer of a hydroxyl group at C-3 of the A-ring of $25(OH)D_3$.

Table 1 ^{1}H-NMR and LC-MS analyses of $25(OH)D_3$ metabolites

Compound	$25(OH)D_3$	3-epi-$25(OH)D_3$	$25(OH)D_3$ metabolite (Peak b)
^{1}H-NMR analysis			
^{1}H chemical shifts and coupling constants[1]			
H-1	2.16 (1H, ddd, *J*=5.0, 8.0, 13.5)	2.13 (1H, ddd, *J*=4.5, 10.0, 14.5)	2.13 (1H, ddd, *J*=4.5, 10.0, 14.5)
	2.38 (1H, ddd, *J*=4.5, 8.0, 13.5)	2.38 (1H, ddd, *J*=5.5, 5.5, 14.0)	2.38 (1H, ddd, *J*=5.5, 5.5, 14.0)
H-2	1.66 (1H, m)	1.66 (1H, m)	1.66 (1H, m)
	1.96 (1H, m)	1.96 (1H, m)	1.96 (1H, m)
H-3	3.93 (1H, m)	3.86 (1H, m)	3.86 (1H, m)
H-6	6.21 (1H, d, *J*=11.5)	6.21 (1H, d, *J*=11.5)	6.21 (1H, d, *J*=11.5)
H-7	6.01 (1H, d, *J*=11.5)	6.02 (1H, d, *J*=11.5)	6.02 (1H, d, *J*=11.5)
H-18	0.52 (3H, s)	0.53 (3H, s)	0.53 (3H, s)
H-19	4.80 (1H, narrow m)	4.82 (1H, narrow m)	4.82 (1H, narrow m)
	5.03 (1H, narrow m)	5.04 (1H, narrow m)	5.04 (1H, narrow m)
H-21	0.92 (3H, d, *J*=6.5)	0.92 (3H, d, *J*=6.5)	0.92 (3H, d, *J*=6.5)
H-26,27	1.19 (6H, s)	1.20 (6H, s)	1.23 (6H, s)
LC-MS analysis			
characteristic ions (m/z)	418.8 $[M+NH_4]^+$	418.8 $[M+NH_4]^+$	418.8 $[M+NH_4]^+$
	401.8 $[M+H]^+$	401.8 $[M+H]^+$	401.8 $[M+H]^+$

[1] Chemical shifts are in ppm; coupling constants are in Hertz (Hz).

Comparison of Metabolism of 25(OH)D_3 among UMR-106, MG-63, Caco-2, LLC-PK_1 and HepG2 Cells.

When 25(OH)D_3 was incubated with cultured cells, 3-epi-25(OH)D_3 and 24,25$(OH)_2D_3$ were observed in all cell lines tested (Fig. 2). In cell cultures of UMR-106, MG-63, Caco-2 and HepG2, 3-epi-25(OH)D_3 was preferentially generated although a small quantity of 24,25$(OH)_2D_3$ was also generated, whereas 24,25$(OH)_2D_3$ and 3-epi-25(OH)D_3 were respectively, major and minor metabolites of 25(OH)D_3 in LLC-PK_1 cells. The amount of 3-epi-25(OH)D_3 generated was greatest in HepG2 cells among the cultures and about 8-fold greater than that of 24,25$(OH)_2D_3$.

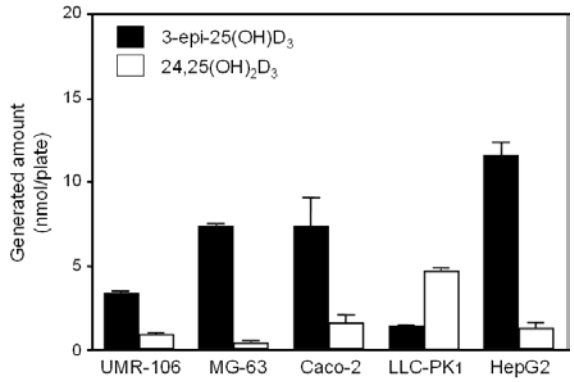

Fig. 2 Relative amounts of 25(OH)D_3 metabolites generated in UMR-106, MG-63, Caco-2, LLC-PK_1 and HepG2 cells. Each cell was incubated with 10 μM of 25(OH)D_3 for 48 h. The results are expressed as the total amount of product formed in nmol/plate/48 h and represent the mean of three experiments (values in columns).

In Vivo Metabolism of 25(OH)D_3 in Rats.

Lipid extracts of the serum obtained from rats intravenously given 500 μg of 25(OH)D_3 were subjected to HPLC and metabolites of 25(OH)D_3 were purified as outlined in the Materials and method section. The major peak corresponding to the authentic standards 25(OH)D_3 and 3-epi-25(OH)D_3 in the first HPLC system was collected in a single fraction, then the eluate was subjected to a second HPLC using a Sumichiral OA-2000 column for

the separation of 3-epi-25(OH)D_3 from 25(OH)D_3. In the second HPLC system, 3-epi-25(OH)D_3 (R.T. 16.68 min) separated from 25(OH)D_3 (R.T. 17.96 min) was observed (data not shown). The calculated serum concentration of 3-epi-25(OH)D_3 was 16.2 ± 6.7 ng/mL (mean ± SE, n=3).

Substrate Specificity of C-3 Epimerization.

Fig. 3 shows the amounts of 3-epi-25(OH)D_3, 3-epi-1α,25(OH)$_2D_3$ and 3-epi-24,25(OH)$_2D_3$ generated from 25(OH)D_3, 1α,25(OH)$_2D_3$ and 24,25(OH)$_2D_3$ in each cell-culture, respectively. In UMR-106, Caco-2 and LLC-PK_1 cells, C-3 epimerization occurred similarly in 25(OH)D_3 and 1α,25(OH)$_2D_3$. In contrast, MG-63 and HepG2 cells epimerized 25(OH)D_3 more preferentially than 1α,25(OH)$_2D_3$. 24,25(OH)$_2D_3$ was hardly epimerized to 3-epi-24,25(OH)$_2D_3$ in any of the cultured cells.

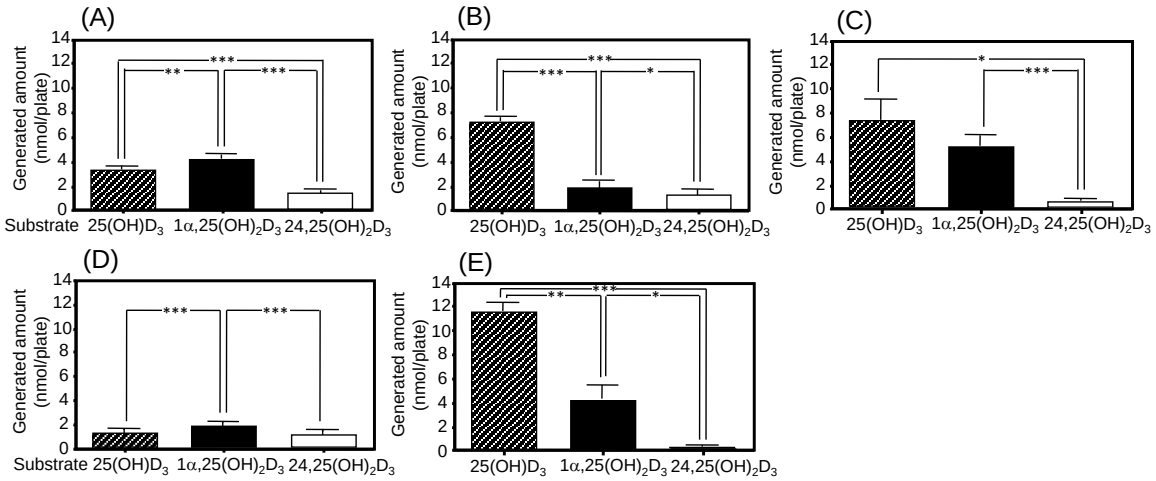

Fig. 3 Relative amounts of C-3 epimers generated from 25(OH)D_3, 1α,25(OH)$_2D_3$ and 24,25(OH)$_2D_3$ in UMR-106, MG-63, Caco-2, LLC-PK_1 and Hep G2 cells. Each cell was incubated with 10 μM of 25(OH)D_3, 1α,25(OH)$_2D_3$ and 24,25(OH)$_2D_3$ for 48 h. The results are expressed as the total amount of product formed in nmol/plate/48h and represent the mean of three experiments (values in columns). (A) UMR-106 cells (B) MG-63 cells (C) Caco-2 cells (D) LLC-PK_1 cells (E) HepG2 cells. Significant difference among the three groups (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Metabolism of 3-Epi-25(OH)D_3 and 3-Epi-1α,25(OH)$_2D_3$ in UMR-106 Cells.

After incubation of 10 μM of 3-epi-25(OH)D_3 or 3-epi-1α,25(OH)$_2D_3$ with UMR-106 cells for 48 h, lipid extracts from the medium along with the cells were subjected to HPLC using a Zorbax SIL column (Fig.4). In the HPLC analysis of 3-epi-25(OH)D_3 metabolites, Peak 2, 3 and 4 in Fig. 4A possessed the characteristic UV chromophore of vitamin D_3 (λmin 228 nm, λmax 265 nm). Peak 4 (R.T. 8.16 min) exhibited the same retention time as the authentic standard of 3-epi-24,25(OH)$_2D_3$ (R.T. 8.14 min). The amount of Peak 4 was significantly increased by pre-incubation with 10 nM 1α,25(OH)$_2D_3$ for 18 h (data not shown). Peak 4 was collected in sufficient quantity to permit identification by ^{1}H-NMR and LC-MS analyses. Peak 1 in Fig. 4A, which possessed the characteristic UV chromophore of previtamin D_3 (λmin 228 nm, λmax 260 nm), was also observed in a control incubation with no cells, only medium. Peak 2 in Fig. 4A containing 3-epi-25(OH)D_3 was collected and purified using a chiral column for the separation of 25(OH)D_3 from 3-epi-25(OH)D_3 as described above. 25(OH)D_3 was not detected by an additional HPLC chromatography under the same conditions (data not shown). Fig. 4B shows the HPLC chromatograms of 3-epi-1α,25(OH)$_2D_3$ metabolites and authentic standards of 1α,25(OH)$_2D_3$, 3-epi-1α,25(OH)$_2D_3$ and 1α,24,25(OH)$_3D_3$. Six peaks possessed the characteristic UV chromophore of vitamin D_3 (λmin 228 nm, λmax 265 nm). Peak 6 in Fig. 4B (R.T. 20.49 min), which was the most prevalent polar metabolite of 3-epi-1α,25(OH)$_2D_3$, was eluted at a less polar position than authentic 1α,24,25(OH)$_3D_3$. Peak 6 in Fig. 4B was collected and purified for ^{1}H-NMR and LC-MS analyses. Peak 4 in Fig. 4B, which eluted at a position approximating that of 1α,25(OH)$_2D_3$ in HPLC using a Zorbax SIL column showed a different retention time from 1α,25(OH)$_2D_3$ in HPLC using a Zorbax CN column (data not shown). These results suggest

that C-3 (α→β) epimerization may be an extremely weak process compared to C-3 (β→α) epimerization or that C-3 (β→α) epimerization is unidirectional.

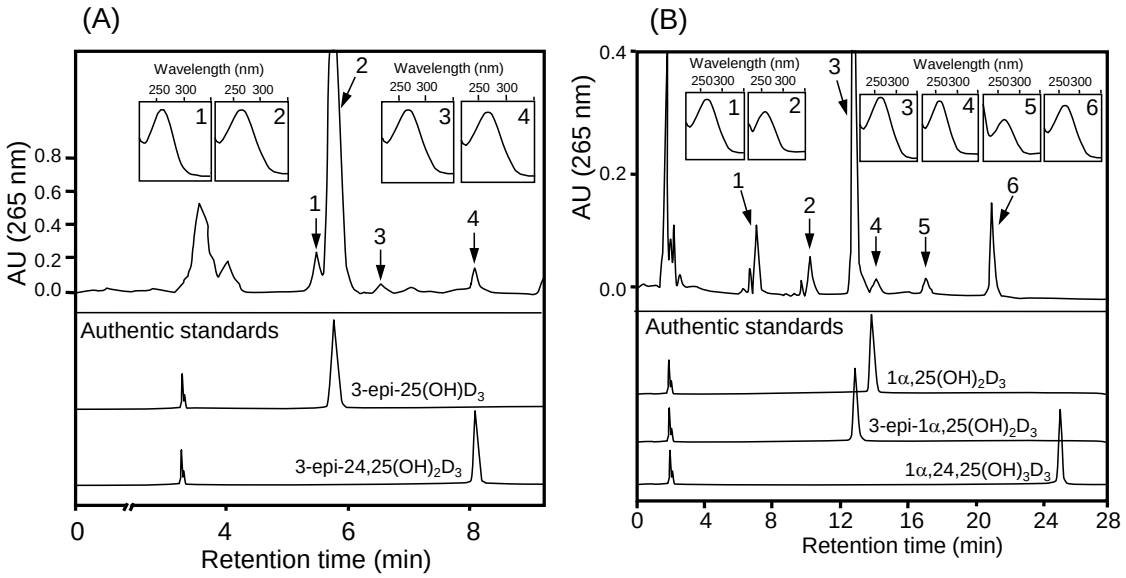

Fig. 4 Metabolism of 3-epi-25(OH)D_3 or 3-epi-1α,25$(OH)_2D_3$ in UMR-106 cells. (A) Upper panel, The HPLC profile and UV spectra of the lipid extracts from UMR-106 cells along with the medium incubated with 10 μM of 3-epi-25(OH)D_3 for 48 h. Lower panel, The HPLC profiles of authentic standards of 3-epi-25(OH)D_3, and 3-epi-24,25$(OH)_2D_3$. (B) Upper panel, The HPLC profile and UV spectra of the lipid extracts from UMR-106 cells along with the medium incubated with 10 μM of 3-epi-1α,25$(OH)_2D_3$ for 48 h. Lower panel, The HPLC profiles of authentic standards of 1α,25$(OH)_2D_3$, 3-epi-1α,25$(OH)_2D_3$, and 1α,24,25$(OH)_3D_3$. HPLC analysis was performed using a Zorbax-SIL column (4.6 × 250 mm) eluted with hexane/2-propanol/methanol (HIM 88/10/2, v/v/v) at a flow rate of 1.0 mL/min.

Identification of 3-Epi-24,25$(OH)_2D_3$ and 3-Epi-1α,24,25$(OH)_3D_3$ by ^{1}H-NMR and LC-MS Analyses.

The ^{1}H chemical shifts assigned based on 1 dimensional (1D) and 2D COSY, NOESY spectra of synthetic standards and 3-epi-25(OH)D_3 metabolite (Peak 4 in Fig. 4A) are summarized in Table 2. The resonances from all protons of the 3-epi-25(OH)D_3 metabolite (Peak 4 in Fig. 4A) completely matched those of authentic 3-epi-24,25$(OH)_2D_3$. In the LC-MS spectra of the 3-epi-25(OH)D_3 metabolite (Peak 4 in Fig. 4A), $[M+H]^+$ and $[M+NH_4]^+$

were observed at *m/z* 417.8 and 434.9 as in the authentic 3-epi-24,25$(OH)_2D_3$. From the findings of ^{1}H-NMR and LC-MS analyses, the 3-epi-25$(OH)D_3$ metabolite (Peak 4 in Fig. 4A) was assigned as 3-epi-24,25$(OH)_2D_3$.

Table 2 ^{1}H-NMR and LC-MS analyses of 3-epi-25$(OH)D_3$ metabolites

Compound	3-epi-25$(OH)D_3$	3-epi-24,25$(OH)_2D_3$	3-epi-25$(OH)D_3$ metabolite (Peak 4 in Fig. 4A)
^{1}H-NMR analysis			
^{1}H chemical shifts and coupling constants[1]			
H-1	2.13 (1H, ddd, *J*=4.5, 10.0, 14.5) 2.38 (1H, ddd, *J*=5.5, 5.5, 14.0)	2.13 (1H, ddd, *J*=4.5, 10.0, 13.5) 2.38 (1H, ddd, *J*=5.0, 5.0, 13.5)	2.13 (1H, ddd, *J*=4.5, 10.0, 13.5) 2.38 (1H, ddd, *J*=5.0, 5.0, 13.5)
H-3	3.86 (1H, m)	3.86 (1H, m)	3.86 (1H, m)
H-6	6.21 (1H, d, *J*=11.5)	6.21 (1H, d, *J*=11.5)	6.21 (1H, d, *J*=11.5)
H-7	6.02 (1H, d, *J*=11.5)	6.02 (1H, d, *J*=11.5)	6.02 (1H, d, *J*=11.5)
H-18	0.53 (3H, s)	0.54 (3H, s)	0.54 (3H, s)
H-19	4.82 (1H, narrow m) 5.04 (1H, narrow m)	4.82 (1H, narrow m) 5.04 (1H, narrow m)	4.82 (1H, narrow m) 5.04 (1H, narrow m)
H-21	0.92 (3H, d, *J*=6.5)	0.92 (3H, d, *J*=6.5)	0.92 (3H, d, *J*=6.5)
H-24	1.0-1.6	3.31 (1H, td, *J*=4.0, 8.0)	3.31 (1H, td, *J*=4.0, 8.0)
H-26,27	1.20 (6H, s)	1.15 (3H, s) 1.20 (3H, s)	1.15 (3H, s) 1.20 (3H, s)
LC-MS analysis			
characteristic ions (m/z)	418.8 $[M+NH_4]^+$ 401.8 $[M+H]^+$	434.9 $[M+NH_4]^+$ 417.8 $[M+H]^+$	434.9 $[M+NH_4]^+$ 417.8 $[M+H]^+$

[1] Chemical shifts are in ppm; coupling constants are in Hz.

The ^{1}H chemical shifts of synthetic standards and 3-epi-1α,25$(OH)_2D_3$ metabolite (Peak 6 in Fig. 4B) are summarized in Table 3. The resonances from all protons of the 3-epi-1α,25$(OH)_2D_3$ metabolite (Peak 6 in Fig. 4B) matched those of authentic 3-epi-1α,25$(OH)_2D_3$ except for H-24, 26 and 27. The chemical shifts of H-24, 26 and 27 of the 3-epi-1α,25$(OH)_2D_3$ metabolite (Peak 6 in Fig. 4B) were observed at the same position as authentic 3-epi-24,25$(OH)_2D_3$. In the LC-MS spectrum of 3-epi-1α,25$(OH)_2D_3$ metabolites (Peak 6 in Fig. 4B), $[M+H]^+$ and $[M+NH_4]^+$ were observed at *m/z* 433.9 and 450.9, indicating an increase of 16 mass units from 3-epi-1α,25$(OH)_2D_3$. Therefore, the 3-epi-1α,25$(OH)_2D_3$ metabolite (Peak 6 in Fig. 4B) was assigned to 3-epi-1α,24,25$(OH)_3D_3$, which is a C-24 hydroxylated metabolite of 3-epi-1α,25$(OH)_2D_3$.

Comparison of Metabolism of 3-Epi-25(OH)D_3 among UMR-106, MG-63, Caco-2, LLC-PK_1 and HepG2 Cells.

The amounts of 3-epi-24,25$(OH)_2D_3$ generated from 3-epi-25(OH)D_3 are shown in Fig. 5. 3-epi-24,25$(OH)_2D_3$ formed in all the cell lines tested. In cell cultures of LLC-PK_1, 3-epi-24,25(OH)D_3 was generated most in five cell lines similar to the production of 24,25$(OH)_2D_3$ from 25(OH)D_3. 3-epi-1α,24,25$(OH)_3D_3$ from 3-epi-1α,25$(OH)_2D_3$ in UMR-106, Caco-2 and LLC-PK_1 cells showed a similar pattern to that in Fig.5 (data not shown).

Table 3 ^{1}H-NMR and LC-MS analyses of 3-epi-1α,25(OH)$_2$D$_3$ metabolites

Compound	3-epi-1α,25(OH)$_2$D$_3$	3-epi-24,25(OH)$_2$D$_3$	3-epi-1α,25(OH)$_2$D$_3$ metabolite (Peak 6 in Fig. 4B)
^{1}H-NMR analysis			
^{1}H chemical shifts and coupling constants[1]			
H-1	4.29 (1H, m)	2.13 (1H, ddd, *J*=4.5, 10.0, 13.5) 2.38 (1H, ddd, *J*=5.0, 5.0, 13.5)	4.29 (1H, m)
H-3	4.03 (1H, m)	3.86 (1H, m)	4.03 (1H, m)
H-6	6.42 (1H, d, *J*=11.0)	6.21 (1H, d, *J*=11.5)	6.42 (1H, d, *J*=11.0)
H-7	6.01 (1H, d, *J*=11.0)	6.02 (1H, d, *J*=11.5)	6.00 (1H, d, *J*=11.0)
H-18	0.53 (3H, s)	0.54 (3H, s)	0.53 (3H, s)
H-19	4.98 (1H, narrow m) 5.28 (1H, narrow m)	4.82 (1H, narrow m) 5.04 (1H, narrow m)	4.98 (1H, narrow m) 5.28 (1H, narrow m)
H-21	0.92 (3H, d, *J*=9.0)	0.92 (3H, d, *J*=6.5)	0.92 (3H, d, *J*=6.5)
H-24	1.0-1.6	3.31 (1H, td, *J*=4.0, 8.0)	3.32 (1H, td, *J*=4.0, 8.0)
H-26,27	1.20 (6H, s)	1.15 (3H, s) 1.20 (3H, s)	1.15 (3H, s) 1.20 (3H, s)
LC-MS analysis			
characteristic ions (m/z)	434.9 $[M+NH_4]^+$ 417.8 $[M+H]^+$	434.9 $[M+NH_4]^+$ 417.8 $[M+H]^+$	450.9 $[M+NH_4]^+$ 433.9 $[M+H]^+$

[1] Chemical shifts are in ppm; coupling constants are in Hz.

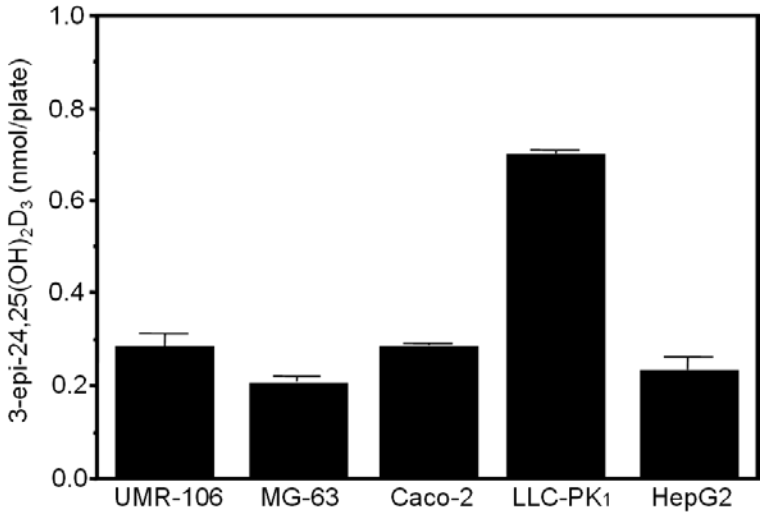

Fig. 5 Relative amounts of 3-epi-24,25$(OH)_2D_3$ generated from 3-epi-25$(OH)D_3$ in UMR-106, MG-63, Caco-2, LLC-PK_1 and HepG2 cells. Each cell was incubated with 10 μM of 3-epi-25$(OH)D_3$ for 48 h. The results are expressed as the total amount of product formed in nmol/plate/48 h and represent the mean of three experiments (values in columns).

Time Course Production of Metabolites of 25$(OH)D_3$, 3-epi-25$(OH)D_3$, 1α,25$(OH)_2D_3$ and 3-epi-1α,25$(OH)_2D_3$.

In order to compare the production of metabolites of 25$(OH)D_3$, 1α,25$(OH)_2D_3$ and their C-3 epimers, UMR-106 cells were incubated with 5 μM of 25$(OH)D_3$ (Fig. 6A), 5 μM of 3-epi-25$(OH)D_3$ (Fig. 6B), 1 μM of 1α,25$(OH)_2D_3$ (Fig. 6C) or 3-epi-1α,25$(OH)_2D_3$ (Fig. 6D) for various periods between 0 and 48 h. In Fig. 6A, 3-epi-25$(OH)D_3$ was detected first approximately 1h after the incubation started. 24,25$(OH)_2D_3$ was observed at 3 h. 3-epi-25$(OH)D_3$ exhibited a constant value at 12 h. The amount of 24,25$(OH)_2D_3$ continued to increase by 48 h, but reached only 40 % of the concentration of 3-epi-25$(OH)D_3$. The amount of 3-epi-24,25$(OH)_2D_3$ generated from 3-epi-25$(OH)D_3$ was much lower than that of 24,25$(OH)_2D_3$ generated from 25$(OH)D_3$ at all time points (Fig. 6B). 3-epi-1α,25$(OH)_2D_3$ and 1α,24,25$(OH)_3D_3$ generated from 1α,25$(OH)_2D_3$ were observed at 6 h into the incubation (Fig. 6C). The amount of 1α,24,25$(OH)_3D_3$ increased by 24 h and then

decreased by 48 h. The amount of 3-epi-1α,24,25(OH)$_3$D$_3$ generated from 3-epi-1α,25(OH)$_2$D$_3$ increased linearly by 48 h (Fig. 6D), but was lower than that of 1α,24,25(OH)$_3$D$_3$ from 1α,25(OH)$_2$D$_3$ by 12 h from the start of incubation.

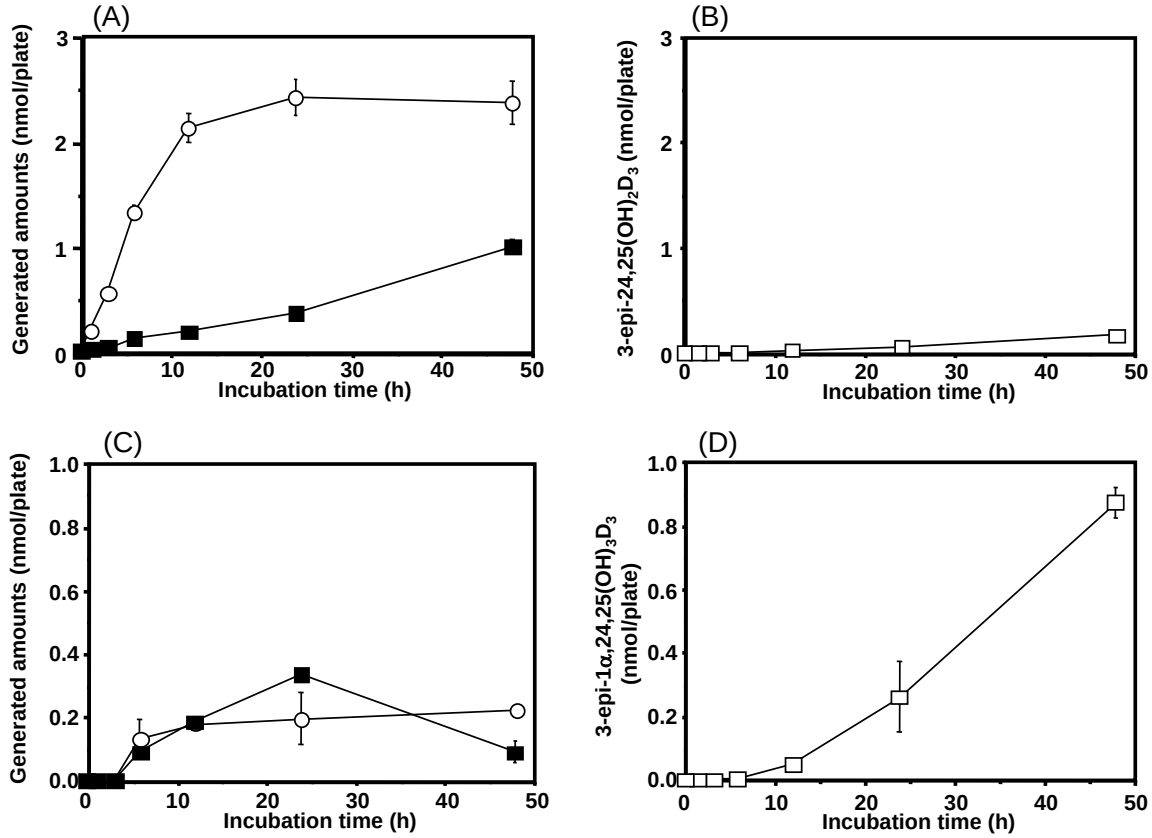

Fig. 6 Time course of 25(OH)D$_3$, 3-epi-25(OH)D$_3$, 1α,25(OH)$_2$D$_3$ and 3-epi-1α,25(OH)$_2$D$_3$ metabolism in UMR-106 cells. (A) Amounts of 25(OH)D$_3$ metabolites in UMR-106 cells incubated with 5 μM of 25(OH)D$_3$ for 1–48 h. Open circle, 3-epi-25(OH)D$_3$; closed square, 24,25(OH)$_2$D$_3$. (B) Amount of 3-epi-25(OH)D$_3$ metabolite in UMR-106 cells incubated with 5 μM of 3-epi-25(OH)D$_3$ for 1–48 h. Open square, 3-epi-24,25(OH)$_2$D$_3$. (C) Amounts of 1α,25(OH)$_2$D$_3$ metabolites in UMR-106 cells incubated with 1 μM of 1α,25(OH)$_2$D$_3$ for 1–48 h. Open circle, 3-epi-1α,25(OH)$_2$D$_3$; closed square, 1α,24,25(OH)$_3$D$_3$. D, Amount of 3-epi-1α,25(OH)$_2$D$_3$ metabolite in UMR-106 cells incubated with 1 μM of 3-epi-1α,25(OH)$_2$D$_3$ for 1–48 h. Open square, 3-epi-1α,24,25(OH)$_3$D$_3$. The results are expressed as the amount of product formed in nmol/plate and represent the mean of three experiments (values in column).

Metabolism of 3-Epi-25(OH)D_3 by CYP27B1 and CYP24A1 Expressed in Recombinant E. Coli Cell-Culture Systems.

After incubation of 50 μM of 25(OH)D_3 with *E. Coli* JM109/pKSNdl, JM109/pKSNdl-CYP27B1 and JM109/pKSNdl-CYP24A1 for 48 h, lipid extracts from the media along with the cells were subjected to HPLC (Fig. 7A). The C-1α-hydroxylated metabolite of 25(OH)D_3, 1α,25(OH)$_2D_3$, was detected in the recombinant JM109/pKSNdl-CYP27B1 cells. 25(OH)D_3 was metabolized to 24,25(OH)$_2D_3$ in JM109/pKSNdl-CYP24 cells. In the control JM109/pKSNdl cells, neither 1α,25(OH)$_2D_3$ nor 24,25(OH)$_2D_3$ was detected. Next, we examined the metabolism of 3-epi-25(OH)D_3 in JM109/pKSNdl, JM109/pKSNdl-

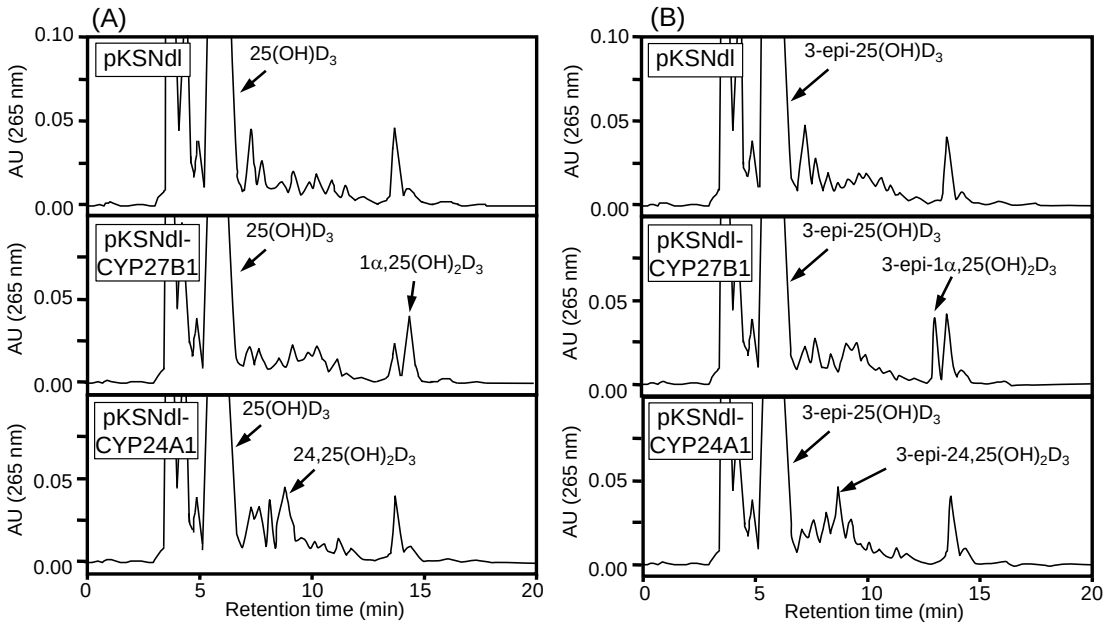

Fig. 7 Metabolism of 3-epi-25(OH)D_3 in cultures of recombinant *E. Coli* cells expressing CYP27B1 and CYP24. (A) The HPLC profile of the lipid extracts from *E. Coli* JM109/pKSNdl, JM109/pKSNdl-CYP27B1 and JM109/pKSNdl-CYP24A1 along with the medium incubated with 50 μM of 25(OH)D_3 for 48 h. (B) The HPLC profile of the lipid extracts from *E. Coli* JM109/pKSNdl, JM109/pKSNdl-CYP27B1 and JM109/pKSNdl-CYP24A1 along with the medium incubated with 50 μM of 3-epi-25(OH)D_3 for 48 h. HPLC analysis was performed using a Zorbax-SIL column (4.6 × 250 mm) eluted with hexane/2-propanol/methanol (HIM 88/10/2, v/v/v) at a flow rate of 1.0 mL/min.

CYP27B1 and JM109/pKSNdl-CYP24A1 (Fig. 7B). 3-epi-25(OH)D_3 was metabolized to polar metabolites with the same retention time as 3-epi-1α,25(OH)$_2D_3$ in JM109/pKSNdl-CYP27B1 cells, and 3-epi-24,25(OH)$_2D_3$ in JM109/pKSNdl-CYP24A1 cells. LC-MS spectra of these metabolites of 3-epi-25(OH)D_3 obtained in JM109/pKSNdl-CYP27B1 and JM109/pKSNdl-CYP24A1 cells completely matched those of synthetic standards of 3-epi-1α,25(OH)$_2D_3$ and 3-epi-24,25(OH)$_2D_3$. 3-epi-25(OH)D_3 was not metabolized to 3-epi-1α,25(OH)$_2D_3$ and 3-epi-24,25(OH)$_2D_3$ in the control JM109/pKSNdl cells. These results suggest that 3-epi-25(OH)D_3 was metabolized through hydroxylation at position C-1α by CYP27B1 or position C-24 by CYP24A1 as well as 25(OH)D_3.

Biological Activities of 1α,25(OH)$_2D_3$, 25(OH)D_3, 24,25(OH)$_2D_3$ and Their C-3 Epimers.

The biological activity of 1α,25(OH)$_2D_3$, 25(OH)D_3, 24,25(OH)$_2D_3$ and their C-3 epimers is summarized in Table 4. The results indicate that the VDR-binding affinity of C-3 epimers is lower than that of 1α,25(OH)$_2D_3$, 25(OH)D_3 and 24,25(OH)$_2D_3$. As expected, 3-epi-25(OH)D_3 and 3-epi-24,25(OH)$_2D_3$ have high binding affinity to DBP like 25(OH)D_3 and 24,25(OH)$_2D_3$. The relative transcriptional activity toward a human osteocalcin gene promoter containing VDRE in transfected MG-63 cells was about 12 % that of 1α,25(OH)$_2D_3$ for 3-epi-1α,25(OH)$_2D_3$. 25(OH)D_3, 24,25(OH)$_2D_3$ and their C-3 epimers exhibited little effect at a high concentration (10^{-6} M). Similar effects of 25(OH)D_3, 24,25(OH)$_2D_3$ and their C-3 epimers on rat CYP24 gene transactivation were also observed. Relative anti-proliferative and differentiation inducing activities were about 29 and 9 % those of 1α,25(OH)$_2D_3$ for 3-epi-1α,25(OH)$_2D_3$. 25(OH)D_3, 24,25(OH)$_2D_3$ and their C-3 epimers failed to exhibit activity to arrest the cell cycle at G0-G1 and induce cell differentiation.

Table 4 Biological activity of C-3 epimers of vitamin D_3 metabolites

	VDR binding affinity[1]	DBP binding affinity[1]	Transcriptional activity		Anti-proliferative activity[2]	Differentiation-inducing activity[2]
			hOC	rCYP24A1		
1α,25(OH)$_2$D$_3$	100	100	100	100	100	100
3-epi-1α,25(OH)$_2$D$_3$	2.1	46	12.1	13.4	29	9.4
25(OH)D$_3$	0.1	9091	1.4	2.6	2.2	1.1
3-epi-25(OH)D$_3$	0.003	3273	<0.5	<0.8	< 1.5	< 0.3
24,25(OH)$_2$D$_3$	0.03	7182	0.9	2.3	3.9	0.9
3-epi-24,25(OH)$_2$D$_3$	0.0005	4364	<0.5	<0.8	< 1.5	< 0.3

[1] The values represent the relative binding affinity to 1α,25(OH)$_2$D$_3$ (expressed as 100), calculated from the concentration of each compound needed to achieve 50% displacement of [^{3}H]-1α,25(OH)$_2$D$_3$ from VDR or DBP.

[2] The values are expressed as percentage activity (at 50% of the dose-response) in comparison with 1α,25(OH)$_2$D$_3$ (expressed as 100% activity)

Discussion

We demonstrated using ^{1}H-NMR and LC-MS techniques that the major vitamin D_3 metabolite, 25(OH)D_3, is metabolized to its C-3 epimer, 3-epi-25(OH)D_3, in cell culture systems. In addition, we confirmed the production of 3-epi-25(OH)D_3 *in vivo* through analysis of the serum obtained from rats intravenously given 25(OH)D_3. It was reported that 1α,25(OH)$_2D_3$ is metabolized *in vitro* and *in vivo* through the C-3 epimerization pathway along with the side-chain oxidation pathways [10–14]. 3-epi-24,25(OH)$_2D_3$ [18, 19] and 3-epi-24,25(OH)$_2D_3$-24-glucronide [17] were identified as C-3 epimerized metabolites of 24,25(OH)$_2D_3$. These findings indicate that the C-3 epimerization of the A-ring appears to be a common metabolic pathway for the major metabolites of vitamin D_3.

We have also demonstrated that C-3 epimers (C-3α) are metabolized through the C-1α hydroxylation or C-24 hydroxylation pathway similar to C-3β compounds. The further metabolism of C-3 epimers has not been fully understood. Reddy et al. [30] reported that 3-epi-1α,25(OH)$_2D_3$ was metabolized to three polar metabolites, 3-epi-1α,24,25(OH)$_3D_3$, 3-epi-24-oxo-1α,25(OH)$_2D_3$ and 3-epi-24-oxo-1α,23,25-trihydroxyvitamin D_3 in human keratinocytes. In this study, we also confirmed that 3-epi-1α,25(OH)$_2D_3$ was metabolized to its C-24 hydroxylated metabolite, 3-epi-1α,24,25(OH)$_3D_3$ and two less polar metabolites possessing a UV spectrum typical of a vitamin D compound consistent with a previous report [30]. A possible structure of either 3-epi-1α(OH)D_3-20,25-cyclic ether or 3-epi-1α(OH)D_3-24,25-epoxide was assigned to one of the less polar metabolites through mass spectrometry [30]. The structure of less polar metabolites of 3-epi-1α,25(OH)$_2D_3$ detected in this study could not be assigned by ^{1}H-NMR spectroscopy due to the unavailability of sufficient amounts of the compounds. The generation of less polar metabolites is of interest as a unique and specific metabolic pathway for vitamin D_3 compounds. In addition, Holick

et al. [31] reported that synthesized tritiated 3-epi-vitamin D_3 was converted to polar metabolites corresponding to 3-epi-25(OH)D_3 and 3-epi-1α,25$(OH)_2D_3$ in a vitamin D-deficient rat. In the present study, we clearly demonstrated that hydroxylation at C-1α and C-24 of both 3-epi-25(OH)D_3 and 25(OH)D_3 is catalyzed by recombinant CYP27B1 and CYP24A1 expressed in *E. Coli*. These findings suggest that both CYP27B1 and CYP24A1 undergo C-1α and C-24 hydroxylation irrespective of the stereochemistry of the C-3 hydroxyl group.

The relative VDR binding affinity, transcriptional activity and anti-proliferative/ differentiation-inducing activity of C-3α compounds were weaker than those of C-3β compounds. 3-Epi-1α,25$(OH)_2D_3$ is biologically the most active of the three C-3 epimers, and shows transcriptional activity and anti-proliferative/differentiation-inducing activity. 3-Epi-24,25$(OH)_2D_3$ is less active than 3-epi-25(OH)D_3. Thus, 25(OH)D_3 appears to be metabolized to 3-epi-25(OH)D_3 and subsequently to a biologically active 3-epi-1α,25$(OH)_2D_3$ or biologically inactive 3-epi-24,25$(OH)_2D_3$ in response to changes in the serum concentration of calcium and PTH.

It was reported that 3-epi-1α,25$(OH)_2D_3$ suppresses PTH secretion in cultured bovine parathyroid cells [11] and induces surfactant phospholipid synthesis in pulmonary alveolar type II cells [32] irrespective of its low binding affinity to VDR. High metabolic stability of 3-epi-1α,25$(OH)_2D_3$ has been proposed as a possible explanation for the PTH-suppressing and surfactant phospholipid-inducing effects of 3-epi-1α,25$(OH)_2D_3$ *in vitro*. In the present study, time course changes in the amount of metabolites generated from C-3α compounds and C-3β compounds were compared. A difference in metabolic rate between 3-epi-25(OH)D_3 and 25(OH)D_3 was observed for up to 48 h after the addition of substrates, and the difference between 3-epi-1α,25$(OH)_2D_3$ and 1α,25$(OH)_2D_3$ was observed as early as 12

h from the start of incubation. The results suggest that the high intracellular concentration of C-3 epimers caused by the slower metabolic rate contributes in part to their specific activity. As to the specific activity of C-3 epimers, it was reported that 3-epi-1α,25(OH)$_2$D$_3$ and synthetic analogs were selective in inducing apoptosis [33, 34]. Furthermore, a relationship between C-3 epimerization of vitamin D$_3$ metabolites and differentiation in Caco-2 cells was pointed out [10]. Thus, the C-3 epimerization pathway is likely to play a role in the regulation of cell differentiation and apoptosis along with the inactivation of vitamin D compounds.

Interestingly, C-3 epimerization activity for vitamin D$_3$ metabolites differed depending on cell type. In UMR-106, MG-63, Caco-2 and HepG2 cells, the amount of 3-epi-25(OH)D$_3$ generated from 25(OH)D$_3$ was larger than that of 24,25(OH)$_2$D$_3$. HepG2 cells derived from liver, in which vitamin D$_3$ was metabolized to 25(OH)D$_3$, produced a high level of 3-epi-25(OH)D$_3$. In contrast, the production of 3-epi-25(OH)D$_3$ was less than that of 24,25(OH)$_2$D$_3$ in LLC-PK$_1$ cells derived from kidney. This finding accords with the report that the C-3 epimerization pathway does not operate in isolated perfused rat kidney [10, 12]. In LLC-PK$_1$ cells, a number of C-24 hydroxylated metabolites of 3-epi-25(OH)D$_3$ and 3-epi-1α,25(OH)$_2$D$_3$ were observed. These results suggest that kidney may work in the metabolism of vitamin D compounds through the C-24 oxidation pathway. In reference to the cell specificity of C-3 epimerization, it was also reported that 1α,25(OH)$_2$D$_3$ was metabolized *via* the C-24 and C-23 oxidation pathways but not *via* the C-3 epimerization pathway in human promyelocytic leukemia (HL-60) cells [35]. From these findings, C-3 epimerization is thought not to be catalyzed by CYP24.

We also investigated the substrate specificity of C-3 epimerization in various cultured cells. 25(OH)D$_3$ was metabolized to its C-3 epimer similar to or more than 1α,25(OH)$_2$D$_3$

in all cells. However, the amount of 3-epi-24,25$(OH)_2D_3$ generated from 24,25$(OH)_2D_3$ was smaller than that of C-3 epimers of 25$(OH)D_3$ or 1α,25$(OH)_2D_3$. These tendencies were prominent in HepG2 cells which mainly metabolize 25$(OH)D_3$ to 3-epi-25$(OH)D_3$, but absent from LLC-PK_1 cells which metabolize 25$(OH)D_3$ to 24,25$(OH)_2D_3$ preferentially. These results indicate that 25$(OH)D_3$ and 1α,25$(OH)_2D_3$ are better substrates than 24,25$(OH)_2D_3$ for C-3 epimerization. In this study, no production of C-3β metabolites [25$(OH)D_3$ and 1α,25$(OH)_2D_3$] from C-3α substrates [3-epi-25$(OH)D_3$ and 3-epi-1α,25$(OH)_2D_3$] was detected. Thus, the C-3 (α→β) epimerization activity was thought to be weaker than the C-3 (β→α) epimerization activity.

More recently, the existence of 3-epi-25$(OH)D_3$ in human serum has been reported by many researchers [36-42]. Engelman et al. [37] reported that mean concentration of 3-epi-25$(OH)D_3$ was 1.3 ng/mL [approximately 4 % of 25$(OH)D_3$] in population-based 303 samples. Interestingly, serum 3-epi-25$(OH)D_3$ was found in over 90 % of pregnant women [40] and in all umbilical cord blood samples [41]. Ooms et.al. [42] also reported that 3-epi-25$(OH)D_3$ contribution was 55% of total 25$(OH)D_3$ in early preterm infants at 3 month of age. From these results, the hypothesis that hepatic immaturity plays a role in 3-epi-25$(OH)D_3$ formation was proposed.

In conclusion, we first identified C-3 epimerization of 25$(OH)D_3$ and further metabolism through the C-1α or C-24 hydroxylation of 3-epi-25$(OH)D_3$. Fig. 8 summarizes a C-3 epimerization pathway for vitamin D_3 metabolites and further metabolic pathways for C-3 epimers.

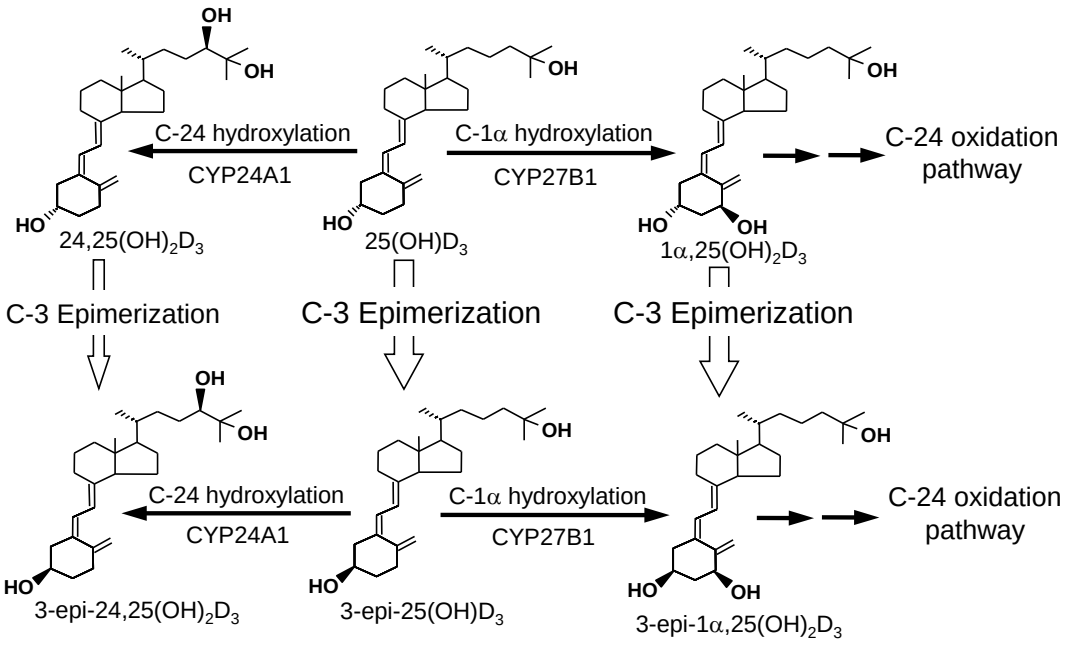

Fig. 8 C-3 epimerization pathway for vitamin D_3 metabolites and further metabolic pathways for C-3 epimers.

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CAPTER 2

Identification of C-3 epimer and C-25 dehydrates of vitamin D analog, 22-oxacalcitriol (maxacalcitriol)

Introduction

The active form of D_3, $1\alpha,25(OH)_2D_3$ plays a crucial role in the regulation of calcium metabolism but it also regulates cell growth and differentiation in normal and malignant cells [1, 2]. In addition, $1\alpha,25(OH)_2D_3$ also inhibits the proliferation of colon [3, 4], breast [5, 6] and prostate cancer cells [7]. However, $1\alpha,25(OH)_2D_3$ will lead *in vivo* to calcemic side effects such as hypercalcemia and hypercalciuria. To overcome this problem, vitamin D analogs with enhanced differentiation/anti-proliferative and reduced hypercalcemic properties have been synthesized. One of them, 22-oxacalcitriol [OCT, 22-oxa-$1\alpha,25(OH)_2D_3$, maxacalcitriol] is a synthetic analog, which has an oxygen atom at position 22, and has received governmental approval for use as an agent for the treatment of secondary hyperparathyroidism and psoriasis in Japan. OCT is rapidly cleared from the circulation due to its extremely low affinity for DBP [8, 9], and binds the chicken VDR with an approximately 8-fold lower affinity than $1\alpha,25(OH)_2D_3$ [10]. However, OCT inhibits growth of psoriatic fibroblasts and enhances the immune response more effectively than $1\alpha,25(OH)_2D_3$ [11, 12]. In contrast, OCT has reduced calcemic effects in both mobilizing calcium from bone and stimulating intestinal calcium transport in normal rats [13]. OCT exhibit different tissue distribution from $1\alpha,25(OH)_2D_3$. After injection of OCT or $1\alpha,25(OH)_2D_3$ into normal rats, the detected amounts of OCT in the parathyroid glands, thymus, adrenals, liver, small intestine, and kidneys were significantly higher than the respective values for $1\alpha,25(OH)_2D_3$, while those of OCT in the plasma and calvaria were significantly lower than those of $1\alpha,25(OH)_2D_3$ [8]. It was also reported that OCT induced interaction of the VDR with a transcriptional intermediary factor (TIF)-2, but not with other transcriptional factors such as steroid receptor coactivator (SRC)-1 and amplified in breast cancer (AIB)-1, while $1\alpha,25(OH)_2D_3$ induced interactions of the VDR with all of the three

co-factors tested [14]. These factors such as metabolic clearance, tissue-specific distribution, cellular uptake, intracellular metabolism and transcriptional regulation could contribute to differences in biological activity.

The metabolism of OCT has been studied in primary parathyroid cells [15] and keratinocytes [16] as well as osteosarcoma, hepatoma and keratin cell lines [17]. In all these systems, OCT is metabolized into hydroxylated and side-chain truncated metabolites, 1α,20-dihydroxyvitamn D_3 [1α,20$(OH)_2D_3$] and hexanor-20-oxo-1α-hydroxyvitamin D_3 [20-oxo-1α$(OH)D_3$]. However, despite relatively large number of products from OCT [17], the structures and properties of the less polar metabolites have not yet been clarified. In the case of natural vitamin D including 1α,25$(OH)_2D_3$, 25$(OH)D_3$ and 24,25$(OH)_2D_3$, one of these less polar metabolites has been identified as C-3 epimers, in which a hydroxyl group at C-3 of the A-ring is epimerized from the β to the α position as described in Part 2, Chapter 1. 3-Epi-1α,25$(OH)_2D_3$ retains 2–3% of the affinity of 1α,25$(OH)_2D_3$ for VDR [18, 19], and exhibits significant activity in reducing growth and promoting differentiation of Caco-2 cells [20].

In this Chapter, we identified the less polar metabolites of OCT including 3-epi-OCT in a rat osteosarcoma cells (UMR-106) and investigated the further metabolism of OCT metabolites. Furthermore, we compared the production rate of OCT metabolites in UMR-106, human colon carcinoma cells (Caco-2) and porcine kidney cells (LLC-PK_1) and examined the biological activity of OCT metabolites. Our findings provide clear evidence that OCT is metabolized to at least three less polar metabolites through two novel pathways, namely C-3 epimerization and C-25 dehydration pathways in addition to the well-known side-chain hydroxylation in target cells.

Materials and methods

Materials.

OCT and its putative metabolite 24-ene-22-oxa-1α-hydroxyvitamin D_3 [24-ene-22-oxa-1α(OH)D_3] were synthesized by Kubodera et al. of Chugai Pharmaceutical Co., Ltd., Japan. 3-Epi-OCT were synthesized by Hatakeyama et al. of Nagasaki University, Japan.

Cell culture.

UMR-106, Caco-2 and LLC-PK_1 cells were maintained as described in "Cell culture" section in Part 2, Chapter 1.

Generation of OCT and 3-epi-OCT metabolites.

For structure assignments, ten plates of UMR-106 cells cultured in 150-mm culture dishes were used. Monolayers were washed with 10 mL of PBS (−) and then incubated in 10 mL of media containing 1% BSA, in the presence of 10 μM OCT or 3-epi-OCT for 48 hr at 37ºC. The no-cell control consisted of 10 mL of the medium and 10 μM OCT or 3-epi-OCT was incubated for the same length of time. For measurement of amounts of OCT and 3-epi-OCT metabolites, three plates each of UMR-106, LLC-PK_1 and Caco-2 cells cultured in 150-mm culture dishes were used. For time-course studies, the cells were incubated with 5 μM of substrates for periods of time ranging from 1 hr to 48 hr. For dose-response studies, the cells were incubated with increasing amounts (0.1–10 μM) of substrates for 24 hr.

Purification of OCT and 3-epi-OCT metabolites by HPLC.

Lipid extraction was performed according to the method described in "Purification Metabolites" section in Part 2, Chapter 1. Separation of metabolites by HPLC was initially

achieved using a Zorbax SIL column (4.6 × 250 mm, Agilent Technologies) eluted with HIM (88:10:2) at a flow rate of 1.0 mL/min. Samples with peaks representing a typical chromophore based on the vitamin D cis-triene structure (λmax=265 nm, λmin=228 nm) were collected and evaporated to dryness, then redissolved in HIM (88:10:2). These were further purified on the same HPLC system using a Zorbax CN column (4.6 × 250 mm, Agilent Technologies) eluted with HIM (88:10:2) at a flow rate of 0.9 mL/min. After further purification with the same previous HPLC system, the metabolites were pure enough for LC-MS and ^{1}H-NMR analyses. Concentrations of the metabolites in ethanol were determined spectrophotometrically using a molar extinction coefficient, ε265=18,000.

^{1}H-NMR and LC-MS analyses.

The 500-MHz ^{1}H-NMR spectra of purified metabolites (2–5 μg) were measured as described in "^{1}H-NMR and LC-MS Analyses" section in Part 2, Section 1. LC-MS analysis was also carried out as described in "^{1}H-NMR and LC-MS Analyses" section in Part 2, Section 1.

VDR and DBP binding assay.

The binding affinity for the VDR and the DBP was tested as described in "VDR and DBP binding assay" section in Part 2, Section 1. For VDR binding assay, following dilution series was used: 1α,25(OH)$_2$D$_3$ (0.0078–64 pg/tube), OCT (0.5–1024 pg/tube), 3-epi-OCT (32–16384 pg/tube), 25-ene-22-oxa-1α(OH)D$_3$ (8–32768 pg/tube), 24-ene-22-oxa-1α(OH)D$_3$ (8–32768 pg/tube). For DBP binding assay, following dilution series was used: 25(OH)D$_3$ (0.003125–16 ng/tube), 1α,25(OH)$_2$D$_3$ (0.24–4000 ng/tube), OCT metabolites (0.06–1000 ng/tube).

Transfection and luciferase activity assay.

Transfection and luciferase activity assay was performed as described in "Transfection and luciferase activity assay" section in Part 2, Section 1. MG-63 cells transfected with luciferase reporter plasmid were incubated with 10^{-9} or 10^{-8} M 1α,25(OH)D_3 or OCT compounds for 48 h.

Anti-proliferative activity assay and cell surface antigen expression analysis.

Anti-proliferative activity was measured as described in "Anti-proliferative activity assay" section in Part 2, Section 1. Cell surface antigen expression analysis was performed as described in "Analysis of cell surface antigen expression" section in Part 2, Section 1. For both analyses, HL-60 cells were incubated with OCT or its metabolites (10^{-9}, 10^{-8} M) for 3 days.

Statistics

Values were calculated as mean±SE. Significance levels were determined by Student's t-test.

Results

Metabolism of OCT in UMR-106 cells.

After incubation of 10 μM OCT with UMR-106 cells for 48 h, 5 metabolites which demonstrated the typical vitamin D chromophore (λmax=265 nm, λmin=228 nm: data not shown) were observed (Fig.1). These metabolites were not formed when OCT was incubated with medium in the absence of cells, or when cells were incubated with medium alone (data not shown). Two of the 5 metabolites, labeled Metabolites 4 and 5, were polar metabolites of OCT and identical to the previously identified 1α,20(OH)$_2$D$_3$ and 24-hydroxy-OCT [24(OH)OCT], respectively [17]. Three other less polar metabolites, labeled Metabolites 1, 2 and 3 have not been isolated and identified to date and were thus purified by extensive rechromatography twice on Zorbax CN and Zorbax SIL for structure assignments.

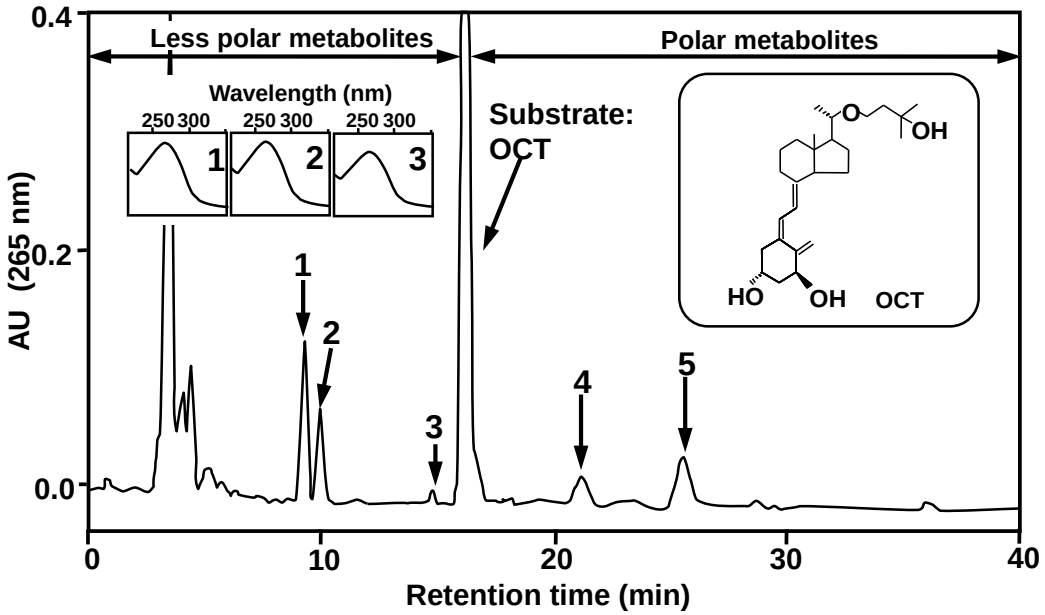

Fig. 1 HPLC chromatograms of the lipid extracts from UMR-106 cells incubated with 10 μM OCT for 48 hr. HPLC analysis was performed using a Zorbax-SIL column (4.6 × 250 mm) eluted with HIM 88:10:2 at a flow rate of 1.0 mL/min. Peaks 1, 2, and 3 were identified as 25-ene-22-oxa-1α(OH)D$_3$, 24-ene-22-oxa-1α(OH)D$_3$ and 3-epi-OCT, respectively.

Identification of less polar metabolites of OCT (Peaks 1, 2 and 3).

Characterizations of OCT Metabolites (Peak 1, 2 and 3) are described below.

Peak 1: The ^{1}H chemical shifts and coupling constants assigned by 1D and 2D COSY and NOESY spectra of Peak 1 are summarized in Table 1. The most pronounced differences between the ^{1}H-NMR spectra of Peak 1 and OCT were found in the 1.6 to 1.8 ppm and 4.6 to 4.8 ppm regions. The disappearance of one of the singlets from the methyl protons in position 26 or 27 (observed at 1.21 and 1.23 ppm in OCT) and the appearance of two singlets at 4.70 and 4.74 ppm, which were assigned to protons of the exo-methylene group, were observed. These findings indicate that dehydration took place at the C-25 hydroxyl group. The resonances from H-24 (2.23 ppm) were shifted downfield compared with those of OCT (1.71 ppm). In the 2D COSY spectrum, cross peaks of the neighboring protons were detected. Connectivity of the H-23 and 23' (3.33 and 3.63 ppm) to the H-24 resonance (2.23 ppm) was observed (Fig. 2A). The other cross-peaks observed in 2D COSY analysis are also shown in Fig. 2A. Peak 1 was assigned to 25ene-22-oxa-1α-hydroxyvitamin D_3 [25-ene-22-oxa-1α(OH)D_3], which has an OCT- like structure with an additional exo-methylene group at the end of the side-chain as a result of dehydration at the C-25 hydroxyl group of OCT. In the LC-MS spectrum of Peak 1, $[M+NH_4]^+$ was observed at m/z 418.6 and indicated a reduction of 18 mass units from OCT (Table 1). Peak 1 gave peaks at m/z 401.6 $[M+H]^+$ and 423.7 $[M+Na]^+$. Therefore, the LC-MS spectrum supports the assignment of Peak 1 structure by ^{1}H-NMR analysis.

Peak 2: The ^{1}H-NMR spectrum of Peak 2 also showed an unchanged structure of ring A, containing the triene system (Table 1). The resonance from H-24 (5.32 ppm) was changed in contrast with that of OCT (1.71 ppm). The intensity of the H-24 signal was found to be

only half of the expected value. Two singlets from the methyl protons in positions 26 and 27 (1.64 and 1.71 ppm) were shifted downfield compared with those of OCT (1.21 and 1.23

Table 1 ^{1}H-NMR and LC-MS analyses of OCT metabolites

Compound	OCT	3-epi-OCT	24-ene-22-oxa-1α(OH)D_3	Peak 1	Peak 2	Peak 3
^{1}H-NMR analysis						
^{1}H chemical shifts and coupling constants[1]						
H-1	4.41 (1H, m)	4.31 (1H, m)	4.41 (1H, m)	4.42 (1H, m)	4.42 (1H, m)	4.31 (1H, m)
H-3	4.21 (1H, m)	4.04 (1H, m)	4.21 (1H, m)	4.21 (1H, m)	4.21 (1H, m)	4.03 (1H, m)
H-6	6.35 (1H, d, *J*=11.5)	6.41 (1H, d, *J*=12.0)	6.36 (1H, d, *J*=11.5)	6.36 (1H, d, *J*=11.0)	6.36 (1H, d, *J*=10.5)	6.41 (1H, d, *J*=11.5)
H-7	6.00 (1H, d, *J*=11.0)	6.00 (1H, d, *J*=11.5)	6.01 (1H, d, *J*=11.5)	6.01 (1H, d, *J*=11.0)	6.01 (1H, d, *J*=11.0)	6.00 (1H, d, *J*=11.5)
H-19	4.97 (1H, narrow m)	4.97 (1H, narrow m)	4.98 (1H, narrow m)	4.98 (1H, narrow m)	4.98 (1H, narrow m)	4.98 (1H, narrow m)
	5.31 (1H, narrow m)	5.27 (1H, narrow m)	5.31 (1H, narrow m)	5.31 (1H, narrow m)	5.31 (1H, narrow m)	5.28 (1H, narrow m)
H-23	3.46 (1H, td, *J*=5.5, 9.5)	3.46 (1H, td, *J*=5.5, 9.0)	3.79 (1H, dd, *J*=7.0, 11.0)	3.33 (1H, td, *J*=7.5, 9.0)	3.79 (1H, dd, *J*=6.0, 10.5)	3.46 (1H, td, *J*=5.5, 8.5)
	3.82 (1H, td, *J*=5.0, 9.5)	3.82 (1H, td, *J*=6.0, 9.0)	4.01 (1H, dd, *J*=6.5, 11.0)	3.63 (1H, td, *J*=7.0, 9.0)	4.01 (1H, dd, *J*=7.0, 11.5)	3.82 (1H, td, *J*=5.5, 9.0)
H-24	1.71 (2H, t, *J*=5.5)	1.71 (2H, t, *J*=5.5)	5.32 (1H, m)	2.23 (2H, td, *J*=6.0, 6.3)	5.32 (1H, m)	1.71 (2H, t, *J*=5.5)
H-26,27	1.21 (3H, s)	1.21 (3H, s)	1.64 (3H, sl)	1.72 (3H, sl)	1.64 (3H, sl)	1.21 (3H, s)
	1.23 (3H, s)	1.23 (3H, s)	1.71 (3H, sl)	4.70 (1H, narrow m)	1.71 (3H, sl)	1.23 (3H, s)
				4.74 (1H, narrow m)		
LC-MS analysis						
characteristic ions (m/z)	441.7 $[M+Na]^+$	441.7 $[M+Na]^+$	423.7 $[M+Na]^+$	423.7 $[M+Na]^+$	423.7 $[M+Na]^+$	441.7 $[M+Na]^+$
	436.7 $[M+NH_4]^+$	436.7 $[M+NH_4]^+$	418.6 $[M+NH_4]^+$	418.6 $[M+NH_4]^+$	418.6 $[M+NH_4]^+$	436.7 $[M+NH_4]^+$
	419.6 $[M+H]^+$	419.6 $[M+H]^+$	401.6 $[M+H]^+$	401.6 $[M+H]^+$	401.6 $[M+H]^+$	419.6 $[M+H]^+$

[1] Chemical shifts are in ppm; coupling constants are in Hz.

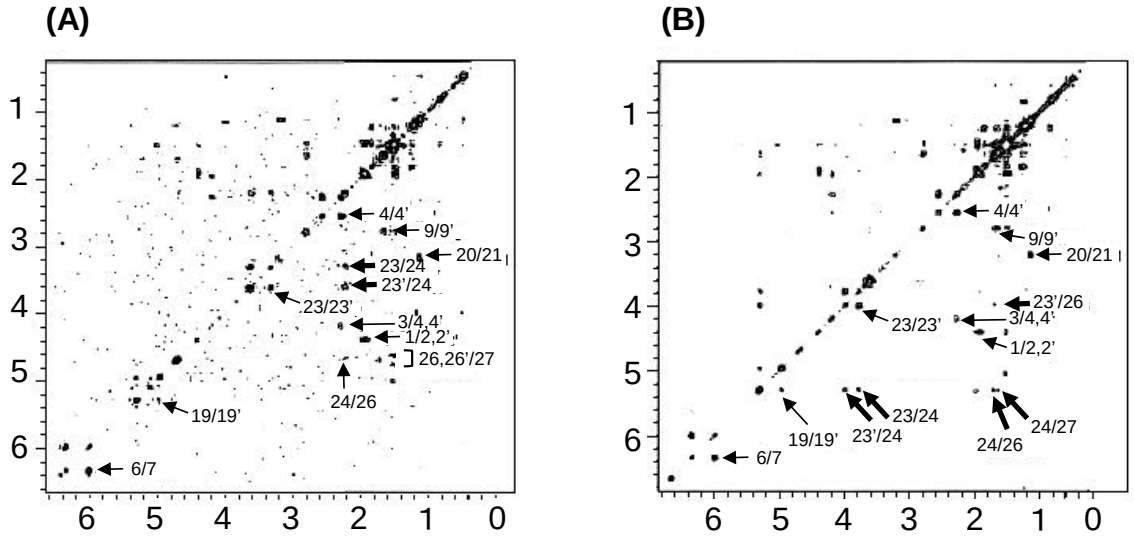

Fig. 2 2D COSY spectrum of OCT Metabolites, Peaks 1 and 2 recorded in $CDCl_3$. (A) Peak 1. (B) Peak 2. The numbers and letters in the spectrum refer to the corresponding protons containing cross-peaks in Peaks 1 and 2.

ppm). The resonances from H-23 and 23' (3.79 and 4.01 ppm) were also shifted downfield. These findings indicate that an olefin group was introduced between C-24 and C-25, and only one proton atom at H-24 remained in Peak 2. In the 2D COSY spectrum, cross peaks showing connectivity between the neighboring protons were clearly observed (Fig. 2B). The signal at 1.71 ppm was assigned to the proton of *trans* position against C-23 (namely, C-26 position) because of the presence of cross-peaks with H-23' and 24 that demonstrate long-range coupling. The signal at 1.64 ppm was also assigned to the proton of cis position against C-23 (namely, C-27 position). The LC-MS spectrum of Peak 2 in principle showed the same features as the spectrum of Peak 1; $[M+NH_4]^+$ was observed at m/z 418.6, suggesting a reduction of 18 mass units from OCT (Table 1). Consequently, Peak 2 was assigned to 24-ene-22-oxa-1α(OH)D_3, a dehydrate of OCT in which a double bond was introduced between the C-24 and C-25 positions by dehydration of the C-25 hydroxyl group of OCT, as was seen in Peak 1. ^{1}H-NMR and LC-MS spectra of synthesized 24-ene-22-oxa-

1α(OH)D were completely congruent with those of Peak 2. Peak 2 co-migrated with authentic 24-ene- 22-oxa-1α(OH)D_3 on Zorbax SIL chromatography.

Peak 3: Peak 3 was inferred to be 3-epi-OCT by co-migration with the authentic standard in HPLC analysis. To confirm this interpretation, ^{1}H-NMR and LC-MS spectral analyses were performed. In the ^{1}H-NMR spectrum of Peak 3, the signals of H-3 (4.03 ppm) and H-1 (4.31 ppm) were observed in the same positions as for authentic 3-epi-OCT, shifted upfield compared with OCT (Table 1). These findings suggest that both protons in positions 1 and 3 tended to be in axial arrangement. In the LC-MS spectra of OCT and 3-epi-OCT, $[M+NH_4]^+$ was observed at m/z 436.7. In the spectrum of Peak 3, $[M+NH_4]^+$ was also observed at m/z 436.7 and the other ions, 419.6 [M+H]+ and 441.7 [M+Na]+ showed identical patterns to the spectra of OCT and 3- epi-OCT respectively (Table 1). It was indicated that the structure of Peak 3 is a diastereomer or a geometric isomer of OCT. Based on the findings of HPLC, ^{1}H-NMR and LC-MS analyses, Peak 3 was assigned as 3-epi-OCT, which has a changed configuration of a hydroxyl group at C-3 of the A-ring.

Metabolism of 3-epi-OCT in UMR-106 cells and identification of less polar metabolites of 3-epi-OCT (Peaks 1' and 2').

Incubation of UMR-106 cells with 10 μM 3-epi- OCT for 48 hr resulted in the formation of 4 metabolites (Fig. 3). Two of the 4 metabolites, labeled Peaks 3' and 4' were corresponded to the C-3 epimers of 1α,20$(OH)_2D_3$ and 24(OH)OCT, respectively. Two other less polar metabolites, labeled Peaks 1' and 2' were purified for structure assignments by ^{1}H-NMR spectroscopy and LC-MS analyses. The proton chemical shifts assigned by 1D and 2D COSY and NOESY spectra of purified Peaks 1' and 2' are summarized in Table 2. Except for the chemical shifts of H-1 and 3, the resonances from all protons of Peaks 1' and

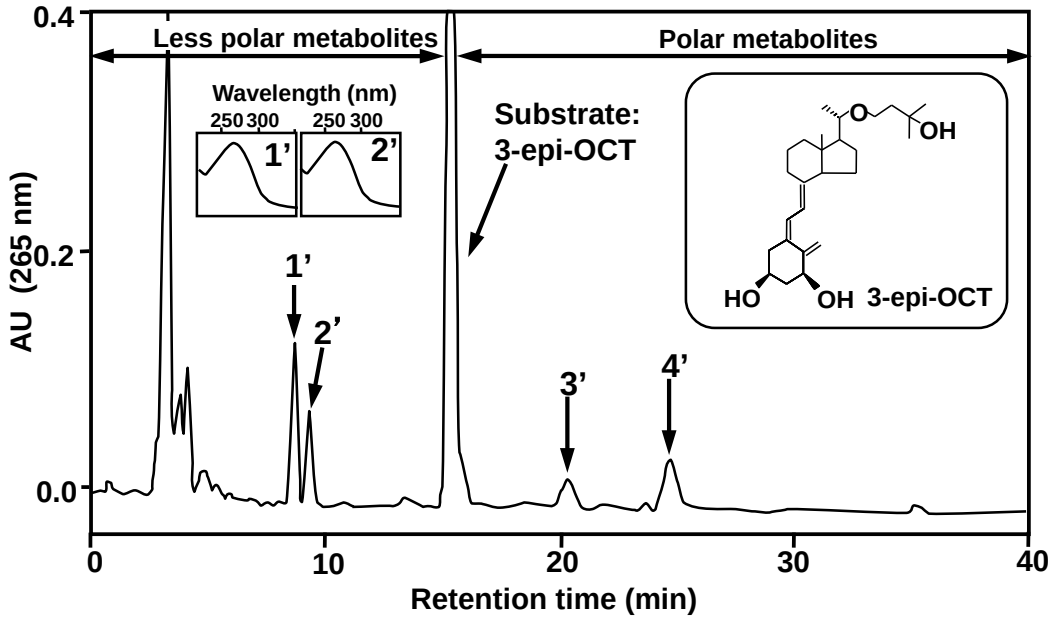

Fig. 3 HPLC chromatograms of the lipid extracts from UMR-106 cells incubated with 10 μM 3-epi-OCT for 48 hr. HPLC analysis was performed using a Zorbax-SIL column (4.6 × 250 mm) eluted with HIM 88:10:2 at a flow rate of 1.0 mL/min. Peaks 1'and 2'were identified as 25-ene-3-epi-22-oxa-1α(OH)D_3 and 24-ene-3-epi-22-oxa-1α(OH)D_3, respectively.

2' matched those of Peaks 1 and 2, respectively. The chemical shifts of H-1 and 3 of Peaks 1' and 2' were observed at the same upfield position as 3-epi-OCT compared to OCT. In the LC-MS spectra of Peaks 1' and 2', $[M+NH_4]^+$ was also observed at m/z 418.6, and the other ions, 401.6 $[M+H]^+$ and 423.7 $[M+Na]^+$ showed identical patterns to the spectra of Peaks 1 and 2 (Table 2). From the findings of ^{1}H-NMR and LC-MS analyses, Peaks 1' and 2' were assigned as 25-ene-3-epi-22-oxa-1α(OH)D_3 and 24-ene-3-epi-22-oxa-1α(OH)D_3, respectively. In this experiment, OCT was not detected as a metabolite of 3-epi-OCT. This finding suggests that C-3 epimerization occurs unidirectionally as previously reported [18, 19].

Table 2 ^{1}H-NMR and LC-MS analyses of 3-epi-OCT metabolites

Compound	OCT	3-epi-OCT	Peak 1	Peak 2	Peak 1'	Peak 2'
^{1}H-NMR analysis						
^{1}H chemical shifts and coupling constants[1]						
H-1	4.41 (1H, m)	4.31 (1H, m)	4.42 (1H, m)	4.42 (1H, m)	4.31 (1H, m)	4.30 (1H, m)
H-3	4.21 (1H, m)	4.04 (1H, m)	4.21 (1H, m)	4.21 (1H, m)	4.05 (1H, m)	4.04 (1H, m)
H-6	6.35 (1H, d, *J*=11.5)	6.41 (1H, d, *J*=12.0)	6.36 (1H, d, *J*=11.0)	6.36 (1H, d, *J*=10.5)	6.41 (1H, d, *J*=11.5)	6.41 (1H, d, *J*=11.5)
H-7	6.00 (1H, d, *J*=11.0)	6.00 (1H, d, *J*=11.5)	6.01 (1H, d, *J*=11.0)	6.01 (1H, d, *J*=11.0)	6.01 (1H, d, *J*=11.5)	6.01 (1H, d, *J*=11.0)
H-19	4.97 (1H, narrow m)	4.97 (1H, narrow m)	4.98 (1H, narrow m)	4.98 (1H, narrow m)	4.98 (1H, narrow m)	4.98 (1H, narrow m)
	5.31 (1H, narrow m)	5.27 (1H, narrow m)	5.31 (1H, narrow m)	5.31 (1H, narrow m)	5.28 (1H, narrow m)	5.28 (1H, narrow m)
H-23	3.46 (1H, td, *J*=5.5, 9.5)	3.46 (1H, td, *J*=5.5, 9.0)	3.33 (1H, td, *J*=7.5, 9.0)	3.79 (1H, dd, *J*=6.0, 10.5)	3.33 (1H, td, *J*=7.5, 9.0)	3.79 (1H, dd, *J*=7.0, 11.0)
	3.82 (1H, td, *J*=5.0, 9.5)	3.82 (1H, td, *J*=6.0, 9.0)	3.63 (1H, td, *J*=7.0, 9.0)	4.01 (1H, dd, *J*=7.0, 11.5)	3.64 (1H, td, *J*=6.5, 9.0)	4.01 (1H, dd, *J*=7.5, 11.5)
H-24	1.71 (2H, t, *J*=5.5)	1.71 (2H, t, *J*=5.5)	2.23 (2H, td, *J*=6.0, 6.3)	5.32 (1H, m)	2.23 (2H, td, *J*=5.0, 6.5)	5.32 (1H, m)
H-26,27	1.21 (3H, s)	1.21 (3H, s)	1.72 (3H, sl)	1.64 (3H, sl)	1.72 (3H, sl)	1.64 (3H, sl)
	1.23 (3H, s)	1.23 (3H, s)	4.70 (1H, narrow m)	1.71 (3H, sl)	4.70 (1H, narrow m)	1.71 (3H, sl)
			4.74 (1H, narrow m)		4.74 (1H, narrow m)	
LC-MS analysis						
characteristic ions (m/z)	441.7 $[M+Na]^+$	441.7 $[M+Na]^+$	423.7 $[M+Na]^+$	423.7 $[M+Na]^+$	423.7 $[M+Na]^+$	441.7 $[M+Na]^+$
	436.7 $[M+NH_4]^+$	436.7 $[M+NH_4]^+$	418.6 $[M+NH_4]^+$	418.6 $[M+NH_4]^+$	418.6 $[M+NH_4]^+$	436.7 $[M+NH_4]^+$
	419.6 $[M+H]^+$	419.6 $[M+H]^+$	401.6 $[M+H]^+$	401.6 $[M+H]^+$	401.6 $[M+H]^+$	419.6 $[M+H]^+$

[1] Chemical shifts are in ppm; coupling constants are in Hz.

Production rates of OCT metabolites in UMR-106, Caco-2 and LLC-PK$_1$ Cells.

The same less polar metabolites of OCT were generated in all cell lines tested, although there were differences exist in the amounts of products formed among cell types (Fig. 4). The major metabolite found in cell cultures of Caco-2 and LLC-PK$_1$ was 24(OH)OCT, whereas 25-ene-22-oxa-1α(OH)D$_3$ appeared to be more prevalent in UMR-106 cells. Interestingly, in UMR-106 cells the production ratio of 25-ene-22-oxa-1α(OH)D$_3$ to 24-ene-22-oxa-1α(OH)D$_3$ was 2:1, however in Caco-2 and LLC-PK$_1$ cells, the production ratios of 25-ene-22-oxa-1α(OH)D$_3$ to 24-ene-22-oxa-1α(OH)D$_3$ were 1:4 and 1:11, respectively.

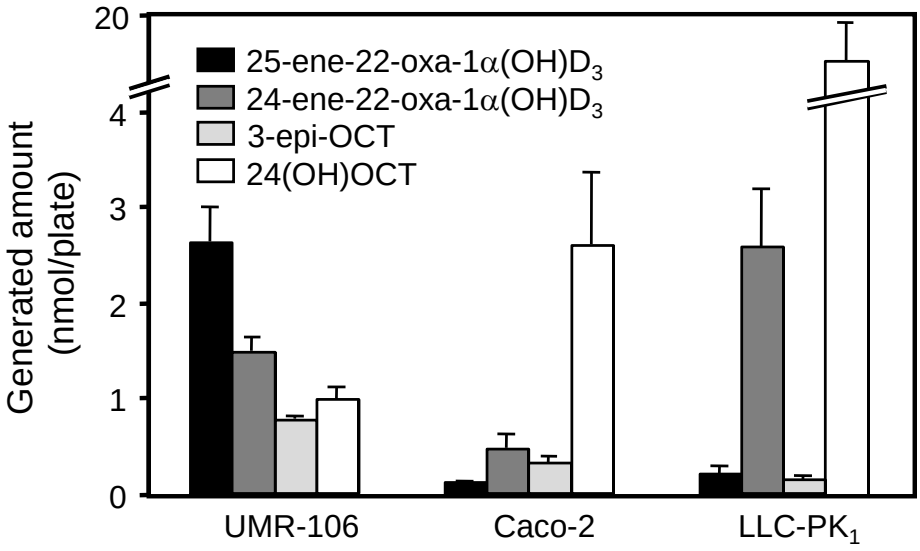

Fig.4. Relative generated amounts of OCT metabolites in UMR-106, Caco-2 and LLC-PK$_1$ cells. Each cell line was incubated with 10 μM OCT for 48 hr. Due to a lack of authentic compounds for 25-ene-22-oxa-1α(OH)D3 and 24(OH)OCT, the amounts of these metabolites were measured by Zorbax SIL HPLC using the metabolites purified from UMR-106 cell culture as the standard compounds. The results are expressed as the total amount of product formed in nmol/plate/48 hr, and represent the mean of three experiments (values in column).

Dose-response studies of metabolism of OCT and 3-epi-OCT in UMR-106 Cells.

25-Ene-22-oxa-1α(OH)D_3, 24-ene-22-oxa-1α(OH)D_3, 24(OH)OCT and 3-epi-OCT were produced in a dose-dependent manner in up to 10 μM OCT as shown in Fig.5A. When UMR-106 cells were incubated with 0.1 μM OCT, 24(OH)OCT was predominantly produced. However, when the cells were incubated with 1-10 μM OCT, the major metabolite was 25-ene-22-oxa-1α(OH)D_3. Interestingly, the production ratio of 25-ene-22-oxa-1α(OH)D_3 to 24-ene-22-oxa-1α(OH)D_3 was 2:1 at any concentration of OCT. We also examined dose-response of less polar metabolism of 3-epi-OCT in UMR-106 cells. Both 25-ene-3-epi-22-oxa-1α(OH)D_3 and 24-ene-3-epi-22-oxa-1α(OH)D_3 were produced in a dose-dependent manner in up to 10 μM 3-epi-OCT (Fig. 5B). At any concentration, the ratio of 25-ene-3-epi-22-oxa-1α(OH)D_3 to 24-ene-3-epi-22-oxa-1α(OH)D_3 was exactly 2:1.

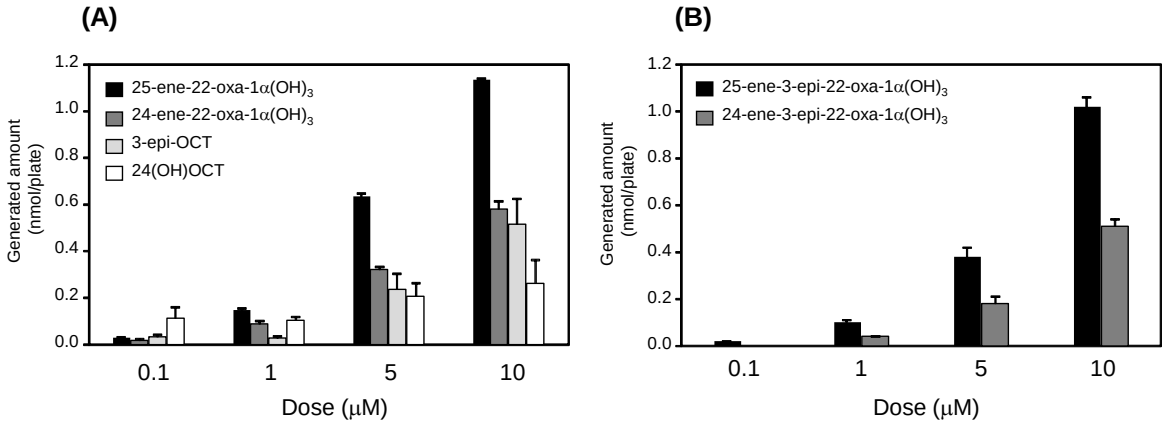

Fig. 5 Dose-response of OCT or 3-epi-OCT metabolism in UMR-106 cells. (A) Amounts of OCT metabolites in UMR-106 cells incubated with 0.1–10 μM OCT for 24 hr. (B) Amounts of the less polar metabolites of 3-epi-OCT in UMR-106 cells incubated with 0.1–10 μM 3-epi-OCT for 24 hr. The results represent the mean of three experiments (values in column).

Time-course studies of metabolism of OCT and 3-epi-OCT in UMR-106 Cells

In a time-course study, 25-ene-22-oxa-1α(OH)D_3 and 24(OH)OCT were first detected approximately 1 h after the incubation was begun, and continued to increase up to the end of the incubation period (Fig. 6A). 24-Ene-22-oxa-1α(OH)D_3 and 3-epi-OCT were first apparent at 3 h of incubation. 24-Ene-22-oxa-1α(OH)D_3 continued to increase up to the end of the incubation period, whereas 3-epi-OCT gradually increased and reached only 30% of the amount of 25-ene-22-oxa-1α(OH)D_3 by the end of the incubation period. The amounts of the two dehydrates generated from 3-epi-OCT in UMR-106 cells, also continued to increase up to the end of the incubation period (Fig. 6B).

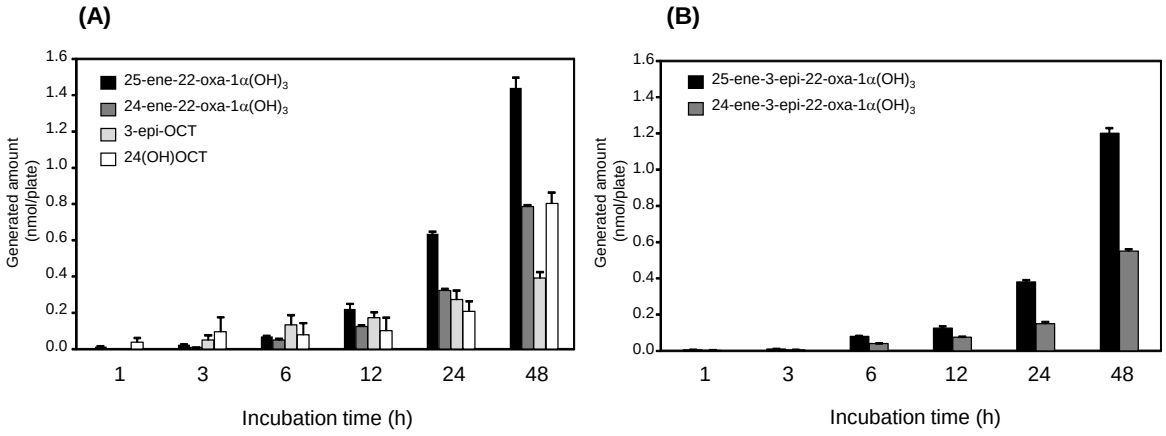

Fig. 6 Time-course of OCT or 3-epi-OCT metabolism in UMR-106 cells. (A) Amounts of OCT metabolites in UMR-106 cells incubated with 5 μM OCT for 1– 48 h. (B) Amounts of the less polar metabolites of 3-epi-OCT in UMR-106 cells incubated with 5 μM 3-epi-OCT for 1–48 hr. The results represent the mean of three experiments (values in column).

Biological activity of OCT and its less polar metabolites

The biological activity of OCT and its less polar metabolites is summarized in Table 3. All of the metabolites tested had a lower binding affinity for VDR than OCT. Relative binding affinities for VDR calculated for 50% displacement of [^{3}H]-1α,25(OH)$_2$D$_3$ were only 0.13, 0.30 and 0.47 % of OCT for 25-ene-22-oxa-1α(OH)D$_3$, 24-ene-22-oxa-1α(OH)D$_3$ and 3-epi-OCT, respectively. All of the metabolites tested had an extremely low binding affinity for DBP, like OCT (data not shown). Relative binding affinities for DBP could not be calculated because all of the metabolites did not reach 50% displacement of [^{3}H]-25(OH)D$_3$. At 10^{-8} M, the transcriptional activities of OCT metabolites on a human osteocalcin gene promoter were about 8, 24 and 33 % of OCT for 25-ene-22-oxa-1α(OH)D$_3$, 24-ene-22-oxa-1α(OH)D$_3$ and 3-epi-OCT, respectively. Similarly, the transcription-inducing activities of OCT metabolites on a rat CYP24 gene promoter were about 2, 11 and 15 % of OCT for 25-ene-22-oxa-1α(OH)D$_3$, 24-ene-22-oxa-1α(OH)D$_3$ and 3-epi-OCT, respectively. Thus, 24-ene-22-oxa-1α(OH)D$_3$ and 3-epi-OCT were found to be less active than OCT, with potencies between 1/3 and 1/10 in terms of the activation of vitamin D-target genes. On the other hand, at 10^{-8} M, no less polar metabolites of OCT showed significant activity of arresting the cell cycle at G_0-G_1 phase as compared to 1α,25(OH)$_2$D$_3$ and OCT (data not shown). Three OCT metabolites had little inducing effect on cell surface CD11b antigen expression in a human promyelocytic leukemia cell line, HL-60. At 10^{-8} M, the biological activities were only 8, 8 and 12 % of OCT for 25-ene-22-oxa-1α(OH)D$_3$, 24-ene-22-oxa-1α(OH)D$_3$ and 3-epi-OCT, respectively.

Table 3 Biological activity of less polar metabolites of OCT

	VDR Binding affinity[1]	Transcriptional activity[2]		Differentiation-Inducing activity[2]
		hOC	rCYP24A1	
OCT	100	100	100	100
25-ene-22-oxa-1α(OH)D_3	0.13	7.3	2.4	13.2
24-ene-22-oxa-1α(OH)D_3	0.30	23.8	10.6	12.4
3-epi-OCT	0.47	32.0	14.9	19.0

[1] The values represent the relative binding affinity to OCT (expressed as 100), calculated from the concentration of each compound needed to achieve 50% displacement of [^{3}H]-1α,25$(OH)_2D_3$ from VDR.

[2] The values are expressed as percentage activity at 10^{-8}M in comparison with OCT (expressed as 100% activity).

Discussion

Here we report the structure assignment of novel less polar metabolites of OCT using ^{1}H-NMR and LC-MS techniques. The novel less polar metabolites identified include two dehydrates [25-ene-22-oxa-1α(OH)D_3 and 24-ene-22-oxa-1α(OH)D_3] and an A-ring diastereomer (3-epi-OCT). In addition, we demonstrated that the 3-epi-OCT was also converted into two dehydrates [25-ene-3-epi-22-oxa-1α(OH)D_3 and 24-ene-3-epi-22-oxa-1α(OH)D_3]. To the best of our knowledge, this is the first definite structural assignment of the C-25 dehydrates of OCT. Siu-Caldera et al. [21] studied the metabolism of 1α,25$(OH)_2D_3$ in UMR-106 and ROS 17/2.8 cells and found a peak corresponding to a less polar metabolite (denoted as M1). Due to the insufficient quantity, its definite structural assignment was not established. If this metabolite M1 is one of the two dehydrates of 1α,25$(OH)_2D_3$, namely 25-ene-1α-hydroxyvitamin D_3 or 24-ene-1α-hydroxyvitamin D_3, then C-25 dehydration would likely be a common metabolic pathway of both 1α,25$(OH)_2D_3$ and OCT.

The formation of these dehydrates was shown to be an enzymatic process, because the metabolites were not observed in no-cell controls incubated with OCT. Two possible metabolic routes from OCT to dehydrates can be considered; one via the direct C-25 dehydration, and the other via some kind of metabolites such as the 24(OH)OCT or 26(OH)OCT. The latter route seems unlikely because the dehydration of hydroxyl groups at positions C-24/C-25 or C-25/C-26 tend to be converted to ketone rather than formation of double bond. It is generally accepted that 24(OH)OCT is metabolized to 24-oxo-OCT, and that 26-hydroxy-OCT [26(OH)OCT] is likely to be metabolized to 23,26-dihydroxy-OCT. Therefore, both 24(OH)OCT and 26(OH)OCT seem unlikely to be precursors of the two OCT dehydrates. The former route is more plausible. Both the 24-ene-22-oxa-1α(OH)D_3

and the 25-ene-22-oxa-1α(OH)D_3 can be formed from OCT by enzymatic C-25 dehydroxylation followed by dehydrogenation at the positions C-24 and C-26,respectively. If the latter oxidation occurs non-enzymatically, then the production of 24-ene-22-oxa-1α(OH)D_3 is expected to be greater than that of 25-ene-22-oxa-1α(OH)D_3 on the basis of the chemical reaction. In this study, unexpectedly, the production of 25-ene-22-oxa-1α(OH)D_3 was greater than that of 24-ene-22-oxa-1α(OH)D_3 in UMR-106 cells. Therefore, the C-25 dehydration process of OCT is suspected to be under strict cell-specific control, or further metabolism of these dehydrates may differ with cell line. The findings of the biological studies here with two dehydrates of OCT demonstrated that their biological activities are considerably lower than OCT. Thus, it appears that like the C-23/C-24 hydroxylation pathways, the C-25 dehydration pathway contributes to reducing the high potency of OCT.

In this study, we also have shown that OCT is metabolized to its C-3 epimer, 3-epi-OCT. The C-3 epimerization is not specific to 1α,25$(OH)_2D_3$, 25(OH)D_3, 24,25$(OH)_2D_3$ (Part 1, Section 1) and OCT. Reddy et al. [22] demonstrated that synthetic vitamin D analogs, 1α,25$(OH)_2$-16-ene-23-yne-vitamin D_3 and 1α,25$(OH)_2$-16-ene-23-yne-20-epi-vitamin D_3 are metabolized to their respective C-3 epimers in UMR-106 cells. These findings clearly indicate that most of the vitamin D derivatives are metabolized through the C-3 epimerization pathway. It is also interesting to note that the rate of C-3 epimerization varies depending upon the structure of vitamin D derivatives. We observed that the rate of C-3 epimerization of 1α,25$(OH)_2D_3$ was about 3-fold higher than that of OCT in UMR-106 cells. In addition, the rate of C-3 epimerization of 1α,25$(OH)_2D_3$ was about 2-fold higher than that of 24,25$(OH)_2D_3$ [23]. Reddy et al. [22] reported that the rate of C-3 epimerization of 1α,25$(OH)_2$-16-ene-23-yne-D_3 was 10-fold lower than that of its C-20 epimer. It was also

found that the rate of C-3 epimerization of 5,6-trans or 19-nor analogs was decreased to significant extent [24]. The vitamin D_3 analogs containing a methyl group at C-2 of the A-ring were not metabolized to their respective C-3 epimers [25]. These findings imply that C-3 epimerization activity is not affected by the C-1a hydroxyl group but by structural modification of the side-chain and A-ring. The C-3 epimerization pathway has been shown to be present in a variety of normal and malignant cells. However, its contribution to the metabolism of vitamin D appears to be relatively low compared to the C-23/C-24 oxidation pathways, except for specific cell lines (e.g. UMR-106 cells). Rat osteosarcoma ROS 17/2.8 cells, in which the C-24 oxidation pathway is not active, has been shown to metabolize $1\alpha,25(OH)_2D_3$ *via* the C-3 epimerization pathway [21]. In contrast, the perfused rat kidney and human promyelocytic leukemia cell line HL-60, in which the C-23/C-24 hydroxylation pathways are highly expressed, do not metabolize $1\alpha,25(OH)_2D_3$ to *via* the C-3 epimerization pathway [21, 26]. These findings imply that C-3 epimerization pathway is cell-selective and contributes to the metabolism of vitamin D in concert with the C-23/C-24 hydroxylation pathways.

In summary, we presented evidence that a novel C-25 dehydration pathway and C-3 epimerization pathway is involved in the metabolism of OCT, a drug for secondary hyperparathyroidism and psoriasis in Japan (Fig. 7). Furthermore, we also demonstrated that 3-epi-OCT is further metabolized to two dehydrates. In UMR-106 cells, OCT is predominantly metabolized via the C-25 dehydration pathway. On the other hand, in Caco-2 and LLC-PK_1 cells, OCT is predominately metabolized via the C-23/C-24 hydroxylation pathways. The interplay of these metabolic pathways may be important in the regulation of OCT metabolism and its biological functions in its target cells.

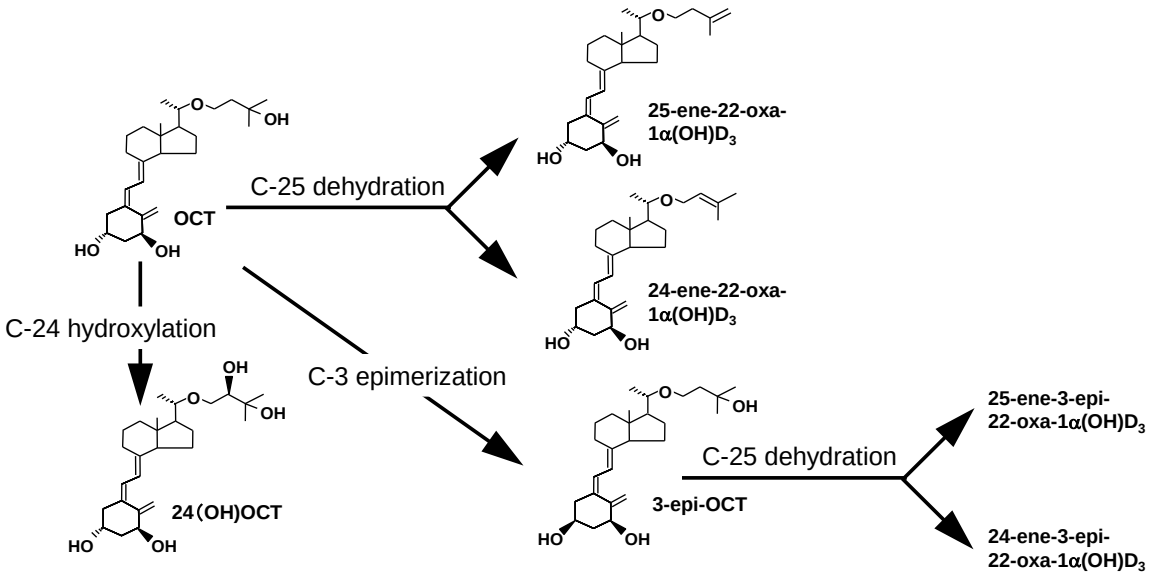

Fig. 7 Novel metabolic pathway of OCT

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CAPTER 3

Measurement of C-3 epimerization activity toward vitamin D

Introduction

Vitamin D is metabolized first in the liver to 25(OH)D_3, and then in the kidney to 1α,25$(OH)_2D_3$ or 24,25$(OH)_2D_3$. It was reported that at least six CYPs can catalyze the initial C-25 hydroxylation step, including CYP2C11 [1–3], CYP2D25 [4,5], CYP2R1 [6,7], CYP2J3 [8], CYP3A4 [9] and CYP27A1 [10–12]. The enzymes engaged in the next C-1α and C-24 hydroxylation steps are CYP27B1 [13–15] and CYP24A1 [16, 17], respectively. Of these enzymes related to vitamin D metabolism, CYP27A1, CYP27B1 and CYP24A1 are located in mitochondria, while CYP2C11, CYP2D25, CYP2R1, CYP2J3 and CYP3A4 are located in microsomes. The properties of mitochondrial enzymes including CYP27A1, CYP27B1 and CYP24A1 have been well studied using *Escherichia coli* expressing these enzymes. Human CYP27A1 produced many metabolites of vitamin D_3, such as C-25, C-26 and C-27 monohydroxide, and C-24,25, C-1α,25 and C-25,26 dihydroxide [18]. These results suggest that human CYP27A1 catalyzes multiple reactions and a multi-step metabolism leading to vitamin D_3. In addition, C-25 hydroxylation to vitamin D_3 showed a smaller maximum velocity (Vmax)/Michaelis constant (Km) value than that to a synthetic analog, 1α-hydroxyvitamin D_3 [1α(OH)D_3] [18]. On the other hand, enzymatic studies on the substrate specificity of CYP27B1 demonstrated that both mouse and human CYP27B1 showed greater Vmax/Km values toward 24,25$(OH)_2D_3$ than 25(OH)D_3 and the C-25 hydroxyl group of vitamin D_3 was essential for the C-1α hydroxylase activity [19, 20]. Rat CYP24 catalyzed a six-step monooxygenation to convert 1α,25$(OH)_2D_3$ into calcitroic acid [21, 22]. It was also demonstrated that human CYP24 catalyzed all the steps of the C-23 oxidation pathway from 25(OH)D_3 to 25(OH)D_3-26,23-lactone in addition to the C-24 oxidation pathway from 25(OH)D_3 to 24,25,26,27-tetranor-23(OH)D_3 [23]. The Vmax/Km values of both rat and human CYP24 were higher for 1α,25$(OH)_2D_3$ than 25(OH)D_3 [21,

23]. CYP24 is highly responsible for the metabolism of both 25(OH)D_3 and 1α,25(OH)$_2D_3$. These enzymes serve important roles in regulating both the formation and distribution of vitamin D metabolites.

In Part 2, Chapter 1 and 2, it has been demonstrated that vitamin D compounds are also metabolized through epimerization at C-3 *in vitro* and *in vivo*. Many of the vitamin D compounds including 1α,25(OH)$_2D_3$ [24, 25], 25(OH)D_3 [26] and 24,25(OH)$_2D_3$ [27, 28], OCT [29], 20-epi-1α,25(OH)$_2D_3$ [30] and 16-ene-23-yne-1α,25(OH)$_2D_3$ [31], have been reported to be metabolized to their respective C-3 epimers. In the case of steroids, the activity that transforms 5α-androstane-3α,17β-diol (C-3α diol) into its C-3 epimer in the rat ovary was reported [32]. The conversion of C-3α hydroxyl group into C-3β might be due to both 3(α→β)-hydroxysteroid epimerase [3(α→β)-HSE] [33] and the combined actions of 3α-hydroxysteroid dehydrogenase (3α-HSD, EC 1.1.1.50) and β-HSD (EC 1.1.1.51). 3(α→β)-HSE, which was cloned in 2000 [33], is a member of the short chain alcohol dehydrogenase family (SDR), and shares high amino acid sequence identity with the retinol dehydrogenases (RoDHs). Higashi et. al. [34] reported that the epimerization of 24,25(OH)$_2D_3$ at C-3 is catalyzed by bacterial 3α-HSD and β-HSD. The conversion of 24,25(OH)$_2D_3$ into 3-epi-24,25(OH)$_2D_3$ and that of 3-epi-24,25(OH)$_2D_3$ into 24,25(OH)$_2D_3$ were both catalyzed by bacterial 3α-HSD and β-HSD, though C-3 epimerization proceeds from C-3β toward C-3α unidirectionally in cultured cells [25, 26, 29]. These observations raise three possibilities: (1) some other enzyme(s) responsible for the C-3 epimerization of vitamin D compounds might exist, (2) the substrate specificity of bacterial 3α-HSD and β-HSD might be different from that of the mammalian enzymes, (3) a mechanism for suppression of epimerization from C-3α to C-3β might operate in mammalian cells.

In the present study, we measured C-3 epimerization activity toward vitamin D_3 in

subcellular fractions and observed the highest level in the microsomal fraction, in which neither 3α-HSD nor β-HSD predominated [35–37]. We evaluated the substrate specificity, effect of cytochrome P450 inhibitors and $1\alpha,25(OH)_2D_3$, and possibility that already-known metabolic enzymes for vitamin D and steroids such as CYP27A1, CYP27B1, CYP24 and 3(α→β)HSE might catalyze the epimerization of vitamin D_3 at C-3.

Materials and methods

Materials.

3-epi-25(OH)D_3, 3-epi-1α,25(OH)$_2D_3$, 3-epi-24,25(OH)$_2D_3$, OCT and 3-epi-OCT [38] were synthesized by Hatakeyama et al. of Nagasaki University. 25(OH)D_3, 1α,25(OH)$_2D_3$ and 24,25(OH)$_2D_3$ were obtained from Solvay Pharmaceuticals B.V. (Veenendaal, The Netherlands). Organic solvents of HPLC grade were purchased from Wako Pure Chemical Industries, Ltd (Tokyo, Japan). Ketoconazole, 1-aminobenzotriazole, benzylimidazole, methoxsalen, metyrapone, SKF-525A, troleandomycin were obtained from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Quinidine was provided by Shigma-Aldorich Co. LLC. (St. Loios, MO, USA). Antiserum against rat NADPH P450 reductase was from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). DNA modifying enzymes, restriction enzymes and the DNA sequencing kit were purchased from Takara Bio Inc. (Kusatsu, Japan). Linker and primer DNAs were obtained from Japan Bio-Service (Saitama, Japan).

Cell culture.

UMR-106, MG-63, Caco-2 and HepG2 were maintained as described in "Cell culture" section in Part 2, Section 1. The human hepatoma cell line (HUH-7) was obtained from the Institute of Development, Aging and Cancer, Tohoku University, Japan. HUH-7 cells were maintained in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10 % FCS and 0.06 mg/mL kanamycin. Cells were cultured at 37 ºC in a humidified atmosphere of CO_2 in air with a change of medium every three days.

Preparation of subcellular fractions.

First, 20 % (w/v) homogenates of cultured cells were prepared in homogenization

buffer [0.25 M sucrose, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2.5 μg/mL leupeptine, 2.5 μg/mL pepstatine and 2 mM dithiothreitol (DTT), pH7.5]. Next, the nuclear fraction was isolated by centrifugation (1,000 × g, 10 min) of the homogenate. The mitochondrial fraction was then isolated by centrifugation (6,500 × g, 20 min) of the supernatant fluid obtained at 1,000 × g. The microsomal fraction was isolated by centrifugation (100,000 × g, 60 min) of a supernatant fluid obtained at 12,000 × g. The pellets were suspended in solubilization buffer [50 mM potassium phosphate, 20 % glycerol, 1 mM ethylenediaminetetraacetic acid, dihydrate (EDTA), 2.5 μg/mL leupeptine, 2.5 μg/mL pepstatine, and 2 mM DTT, pH7.25]. The amount of protein in each fraction was determined with a Pierce BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), using BSA as a standard.

Measurement of C-3 epimerization activity in subcellular fractions.

In the standard incubation procedure, 10 nmol of vitamin D compound dissolved in 5 μL of ethanol was incubated with 4 mg of the subcellular fraction and a NADPH-generating system consisting of 10 μmol of nicotinamide adenine dinucleotide phosphate (NADP), 70 μmol of glucose 6-phosphate, 12 units of glucose 6-phosphate dehydrogenase, and 100 mmol of Mg^{2+}. The incubation volume was adjusted to 1.0 mL with 50 mM potassium phosphate buffer pH 6.5. Incubations were performed at 37 ºC for 60 min and were terminated by addition of 2.5 mL of methanol together with 250 ng of vitamin D_3 or 25(OH)D_3 for the correction of extraction efficiency.

Extraction and purification of metabolites.

Lipid extraction was performed according to the method described in "*Purification of Metabolites"* section in Part 2, Chapter 1.

Measurement of C-3 epimerization activity in cultured cells pre-treated with 1α,25(OH)$_2$D$_3$

UMR-106 cells (2×10^6 cells) were seeded in 150-mm culture dishes and cultured for 4 days to late log phase. At near confluence, 10 nM 1α,25(OH)$_2$D$_3$ was added to the culture medium. After 18 h, the medium was removed and replaced with fresh culture medium supplemented with 1 % BSA. The cells were then incubated for 48 h at 37 ºC in the presence of 1 μM of 25(OH)D$_3$. The extraction of lipids and purification of metabolites were performed as described in the "*Extraction and purification of metabolites*" section.

Measurement of enzyme activity of CYP27A1, CYP27B1, and CYP24

The construction of co-expression plasmids for human CYP27A1, CYP27B1, CYP24 and bovine ADX and NADPH-ADR and culture of recombinant *E. coli* cells were performed as described in "*Metabolism of 3-Epi-25(OH)D$_3$ in Cultures of Recombinant E. Coli Cells Expressing CYP27B1 and CYP24A1"* section in Part 2, Chapter 1. Subcellular fractionation of *E. coli* cells was carried out basically according to previous study [21]. A 100 mM Tris-HCl buffer (pH 7.4) was used for suspension of the membrane fraction. The enzyme activity was measured in a re-constituted system consisting of the membrane fraction containing 0.5 μM of CYP27A1, CYP27B1, and CYP24, 5.0 μM of ADX, 0.5 μM of ADR, 50 μM of 25(OH)D$_3$, 0.5 mM of NADPH, 100 mM Tris-HCl (pH 7.4) and 1 mM EDTA at 37 ºC for 60 min. Lipid extraction and purification of metabolites were performed

as described in the “Extraction and purification of metabolites” section. Recombinant *E. coli* cells transfected with pKSNdl derived from pKK233-3 (JM109/pKSNdl) were used for control experiments.

Measurement of enzyme activity of 3(α→β)HSE

Human 3(α→β)HSE cDNA was obtained from the human liver cDNA library HL1145y (Clontech, Mountain View, CA, USA) using a PCR based method. The oligo-nucleotides CATATGTGGCTCTACCTGGCGGCCTTCGTG and TAAGCTTAGACTGCCTGCCTGGGCTGGTTTG were used as PCR primers on the basis of the human 3(α→β)HSE cDNA sequence described by Huang et. al [33]. The restriction sites *Nde*I and *Hind*III were added to the oligoprimers for subsequent subcloning. The PCR product was ligated with pUC19 digested with *Hinc*II. *E. coli* DH5α (Takara Bil Inc., Kusatsu, Japan) was used as a host strain. The plasmid with the appropriately sized insert was sequenced by using FITC-labeled primers and DSQ-2000L (Shimadzu, Kyoto, Japan). The resultant plasmid was digested with *Xba*I and *Hind*III to yield a *Xba*I-*Hind*III fragment (1.0 kbp). The *Xba*I-*Hind*III fragment was inserted into *Xba*I and *Hind*III sites of the mammalian expression vector pcDNA3.1(−) (Thermo Fisher Scientific Inc., Waltham, MA, USA) to construct pcDNA3.1(−)-3(α→β)HSE. Cos-7 cells (kidney, SV40 transformed, African green monkey, ATCC) were maintained in DMEM supplemented with 10 % FCS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cells (8×10^5) were suspended in 8 mL of the medium and transfected with 10 μg of pcDNA3.1(−)-3(α→β)HSE and pRL-CMV vector (pGVB vector, Toyo Ink Co., Ltd., Tokyo, Japan) as an internal control. The transfection agent used was Tfx-50 reagent (Promega Corp. Madison, WI). The cells were incubated with 5 μM of 25(OH)D_3, 3-epi-25(OH)D_3, androsterone (ADT) or epi-ADT for 48 h at 37ºC in medium containing 1 % BSA. For measurements of vitamin D_3 metabolites,

lipid was extracted and metabolites were purified as described in the "Extraction and purification of metabolites" section. The extracted ADT and epi-ADT metabolites were resuspended in 15 μL of ethanol, applied to Kieselgel-60 high performance thin layer chromatography (HPTLC) plates (Merck, Darmstadt, Germany), and separated by migration in toluene/methanol (9:1, v/v). The metabolites were detected by heating at 120ºC for 15 min after nebulization of sulfuric acid/methanol (1:1, v/v).

Statistical Analysis.

Significance levels were determined by Student's t-test; a value of $p<0.05$ was considered statistically significant.

Results

Optimization of assay conditions for C-3 epimerization activity.

At first, the effect of different co-factors on C-3 epimerization activity was evaluated. In the homogenate prepared from UMR-106 cells, the highest level of activity was observed in the presence of a NADPH-generating system containing glucose-6-phosphate, NADP, glucose-6-phosphate dehydrogenase and Mg^{2+} (data not shown). As shown in Figs.1A and 1B, the rate of production of 3-epi-1α,25$(OH)_2D_3$ was found to be linear with homogenate protein up to about 5 mg and with time for 60 min. As shown in Fig.1C, the optimal pH was between 6.0 and 7.0 and the conversion was somewhat higher in potassium phosphate buffer than acetate-NaOH, MES-NaOH, HEPES-NaOH or Tris-HCl buffer. Thus, the standard incubation procedure for measurement of C-3 epimerization activity was set up the condition using 4 mg of protein and a NADPH-generating system with potassium phosphate buffer, pH 6.5.

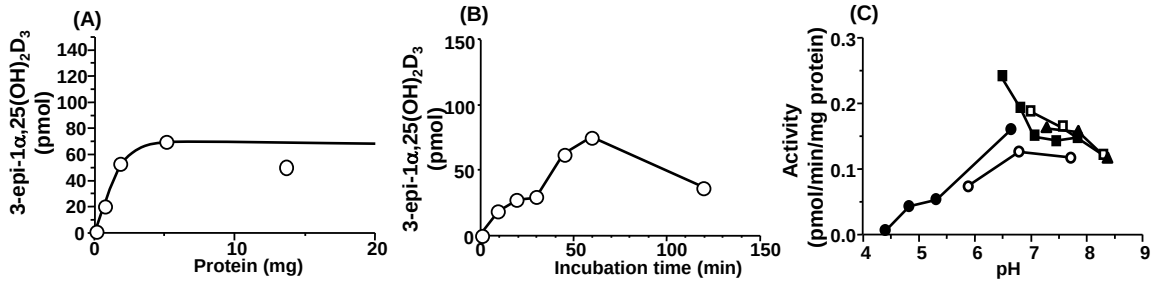

Fig. 1 Effect of protein concentration, incubation time and pH on C-3 epimerization activity toward 1α,25$(OH)_2D_3$ in homogenate prepared from UMR-106 cells. (A) Effect of protein concentration. 10 nmol of 1α,25$(OH)_2D_3$ was incubated with homogenate protein prepared from UMR-106 cells and a NADPH-generating system consisting of 10 μmol of NADP, 70 μmol of glucose 6-phosphate, 12 IU of glucose 6-phosphate dehydrogenase, and 100 mmol of Mg^{2+} in 50 mM potassium phosphate buffer (pH 7.5) at 37 ºC for 60 min. (B) Effect of incubation time. 10 nmol of 1α,25$(OH)_2D_3$ was incubated with 4 mg of homogenate protein prepared from UMR-106 cells and the NADPH-generating system in 50 mM potassium phosphate buffer (pH 7.5) at 37 ºC. (C) Effect of pH. 10 nmol of 1α,25$(OH)_2D_3$ was incubated with 4 mg of homogenate protein prepared from UMR-106 cells and the NADPH-generating system in various buffers at 37 ºC for 60 min. Closed circle, 50 mM Acetate-NaOH buffer; open circle, 50 mM MES-NaOH buffer; closed square, 50 mM potassium phosphate buffer; open square, 50 mM HEPES-NaOH buffer; closed triangle, 50 mM Tris-HCl buffer.

C-3 epimerization activity in subcellular fractions prepared from cultured cells.

As shown in Fig. 2A, when subcellular fractions prepared from UMR-106 cells were incubated with 1α,25(OH)$_2$D$_3$ under standard conditions, the C-3 epimerization activity was strongest in the microsomal fraction. The level of activity in the microsomal fraction was about 60-fold higher than that in the homogenate. In addition, activity was observed in the microsomal fractions prepared from UMR-106, MG-63, Caco-2, HepG2 and HUH-7 cells, respectively (Fig. 2B).

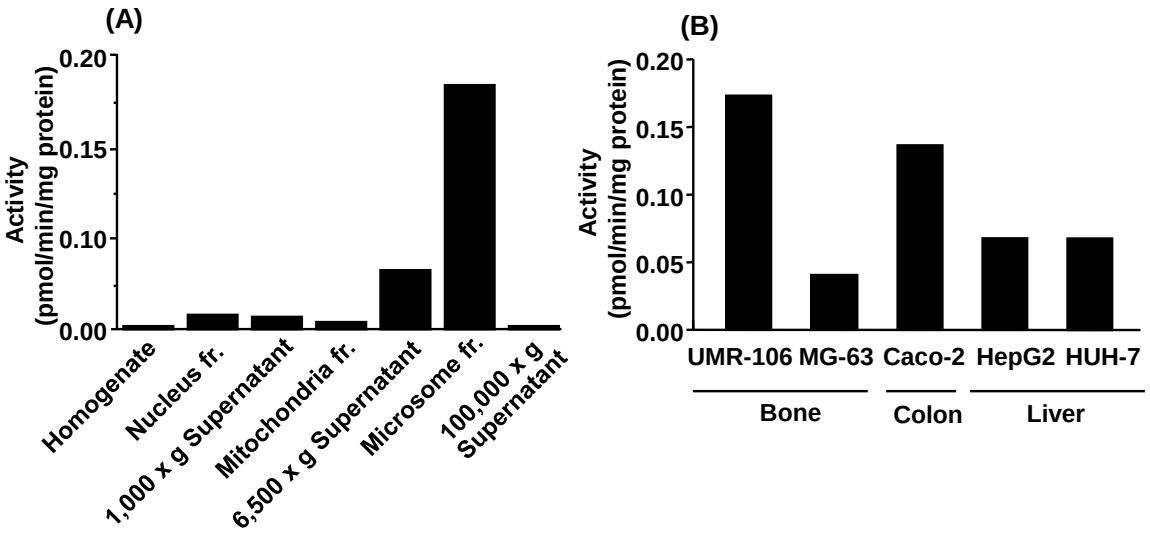

Fig. 2 C-3 epimerization activity toward 1α,25(OH)$_2$D$_3$ in subcellular fractions. (A) C-3 epimerization activity in subcellular fractions prepared from UMR-106 cells under standard conditions. (B) C-3 epimerization activity in microsomal fractions prepared from UMR-106, MG-63, Caco-2, HepG2 and HUH-7 cells under standard conditions. The results represent the mean of two experiments.

Kinetic parameters for C-3 epimerization activity toward vitamin D$_3$ metabolites and an analog.

The major metabolites of D$_3$, 25(OH)D$_3$ and 24,25(OH)$_2$D$_3$, were both metabolized to their C-3 epimers in the microsomal fraction prepared from UMR-106 cells. OCT, a synthetic analog of vitamin D, was also metabolized to its C-3 epimer, however the activity

was quite week. When the substrate concentration was varied, the reaction followed Michaelis-Menten type kinetics for C-3 epimerization. Lineweaver-Burk plots and the calculated Vmax and Km values for the epimerization activity toward vitamin D_3 metabolites and the analog are shown in Fig. 3 and Table 1. The Vmax values was higher for 25(OH)D_3 than for 1α,25(OH)$_2D_3$, 24,25(OH)$_2D_3$ and OCT. The Km was lower for OCT than for 25(OH)D_3, 1α,25(OH)$_2D_3$ and 24,25(OH)$_2D_3$. The physiologically essential parameter Vmax/Km was highest for 25(OH)D_3 of all substrates tested, and about 2-fold higher than that for 1α,25(OH)$_2D_3$. Vmax/Km values for 24,25(OH)$_2D_3$ and OCT were quite low. The microsomal fraction prepared from UMR-106 cells displayed almost no activity for the epimerization of 3-epi-1α,25(OH)$_2D_3$, 3-epi-25(OH)D_3 and 3-epi-24,25(OH)$_2D_3$ to their C-3β form (data not shown).

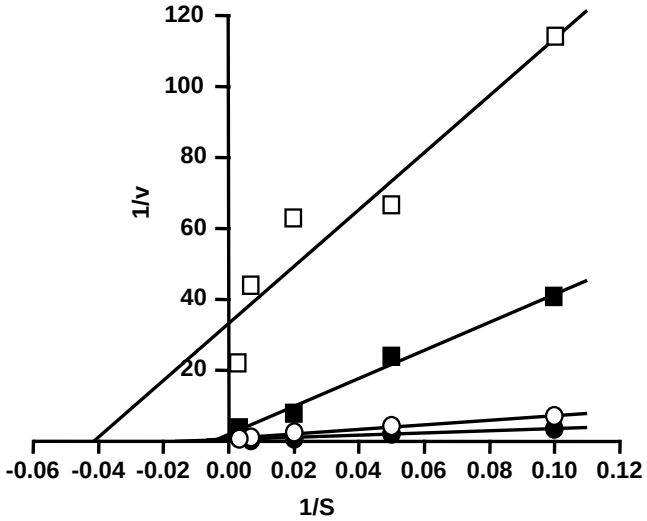

Fig. 3 Lineweaver-Burk plots of 1/V versus 1/S for C-3 epimerization activity toward 25(OH)D_3, 1α,25(OH)$_2D_3$, 24,25(OH)$_2D_3$ and OCT. The C-3 epimerization activity was measured in the microsomal fraction prepared from UMR-106 cells under standard conditions. Closed circle, 25(OH)D_3; open circle, 1α,25(OH)$_2D_3$; closed square, 24,25(OH)$_2D_3$; open square, OCT. S, substrate concentration; V, velocity.

Table 1 Kinetic parameters for C-3 epimerization activity toward D_3 metabolites and an analog

	25(OH)D_3	1α,25(OH)$_2D_3$	24,25(OH)$_2D_3$	OCT
Vmax (pmol/min/mg protein)	2.34	1.52	0.51	0.03
Km (μM)	73.7	98.9	200.1	24.2
Vmax/Km (pmol/min/mg protein/mM)	0.032	0.015	0.0025	0.0012

Effect of various cytochrome P450 inhibitors, antiserum against NADPH P450 reductase on C-3 epimerization activity.

Fig. 4A shows the effect of various cytochrome P450 inhibitors on C-3 epimerization activity. The activity toward 25(OH)D_3 in the microsomal fraction prepared from UMR-106 cells was not inhibited by ketoconazole, 1-aminobenzotriazole, benzylimidazole, methoxalen, metyrapone or SKF-525A, which are categorized as universal cytochrome P450 inhibitors. In addition, the activity was not inhibited by quinidine or troleandomycin, which are known as potent inhibitors of CYP2D6 or CYP3A4, respectively. Fig. 4B shows the effect of antiserum against NADPH P450 reductase. The C-3 epimerization activity was not inhibited by antiserum against NADPH P450 reductase.

Effect of pre-treatment with 1α,25(OH)$_2D_3$ on C-3 epimerization activity.

Figs. 5A and 5B show C-3 epimerization activity in cultured cells pre-treated with 1α,25(OH)$_2D_3$. Pre-treatment with 1α,25(OH)$_2D_3$ did not induce the generation of 3-epi-25(OH)D_3 from 25(OH)D_3 (Fig. 5A), but induced the generation of 24,25(OH)$_2D_3$ from 25(OH)D_3 (Fig. 5B) in UMR-106 cells. These results suggest that the C-3 epimerization was not induced by 1α,25(OH)$_2D_3$.

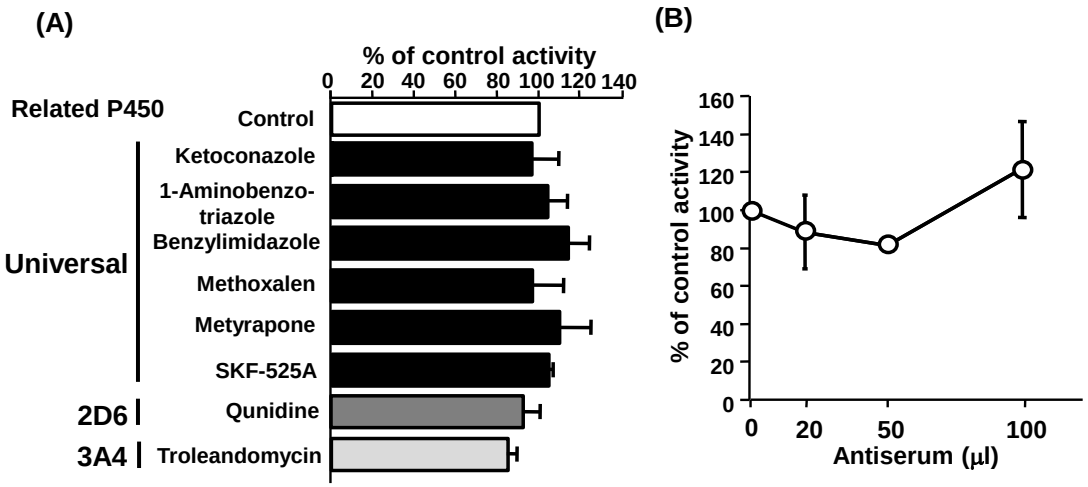

Fig. 4 Effect of various cytochrome P450 inhibitors and antiserum against NADPH P450 reductase on C-3 epimerization activity. (A) Effect of cytochrome P450 inhibitors. The C-3 epimerization activity toward 25(OH)D_3 was measured in the microsomal fraction prepared from UMR-106 cells under standard conditions in the presence of 20 μM of each inhibitor. (B) Effect of antiserum against NADPH P450 reductase. The C-3 epimerization activity toward 25(OH)D_3 was measured in the microsomal fraction prepared from UMR-106 cells under standard conditions. The results represent the mean±SE of three experiments. There were no statistically significant differences from control group.

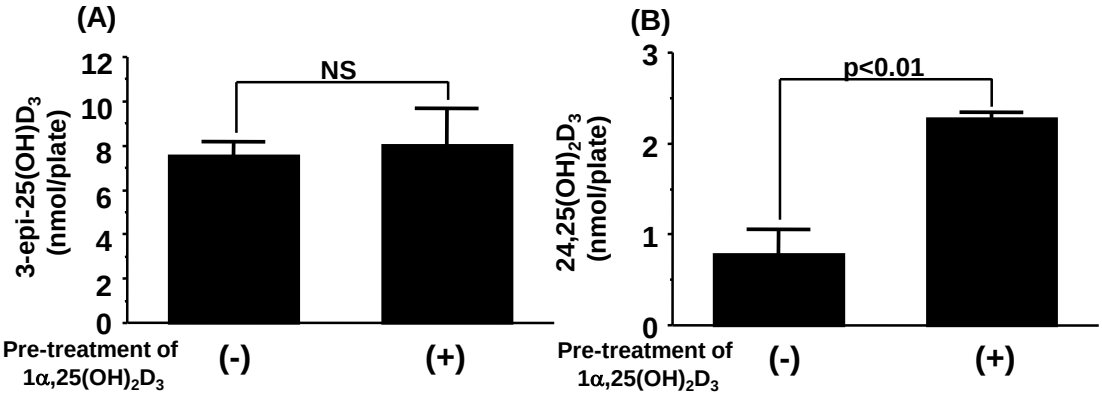

Fig. 5 Effect of pre-treatment with 1α,25(OH)$_2$D$_3$ on C-3 epimerization activity. (A) Relative amounts of 3-epi-25(OH)D$_3$. (B) Relative amounts of 24,25(OH)$_2$D$_3$. 3-epi-25(OH)D$_3$ metabolites generated in UMR-106 cells pre-treated with or without 1α,25(OH)$_2$D$_3$ for 18 h. The cells were incubated with 1 μM of 25(OH)D$_3$ for 48 h. The results are expressed as the total amount of product formed in nmol/plate/48 h and represent mean±SE of three experiments. Significant difference between pre-treatment (-) and pre-treatment (+). NS, not significantly different.

Metabolism of 25(OH)D_3 by CYP27A1, CYP27B1, CYP24 and 3(α→β)HSE.

The metabolism of 25(OH)D_3 was examined in a reconstituted system containing the membrane fraction prepared from the recombinant *E. coli* cells expressing CYP27A1, CYP27B1 or CYP24 with ADX and ADR. The lipid extracts from the reaction mixture of CYP27A1, CYP27B1 and CYP24 were subjected to a first HPLC using a Zorbax SIL column (Fig. 6A). The eluates corresponding to 25(OH)D_3 (R.T. 5.68 min) and 3-epi-25(OH)D_3 (5.68 min) were collected in a single fraction eluting between 5 and 7 min, and then subjected to a second HPLC using a Sumichiral OA-2000 column for the separation of 3-epi-25(OH)D_3 (R.T. 18.23 min) from 25(OH)D_3 (19.72 min) (Fig. 6B). 1α,25$(OH)_2D_3$ was detected as a metabolite of 25(OH)D_3 in the reconstituted system of CYP27A1 and CYP27B1 (Fig. 6A). In the reconstituted system of CYP24, the production of 24,25$(OH)_2D_3$ from 25(OH)D_3 was confirmed as expected. LC-MS spectra of these metabolites of 25(OH)D_3 obtained in the reconstituted system of CYP27A1, CYP27B1 and CYP24 completely matched those of synthetic standards of 1α,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ (data not shown). However, 3-epi-25(OH)D_3 was not produced as a metabolite of 25(OH)D_3 by any of the cytochrome P450 enzymes tested (Fig. 6B). Next, we examined the metabolism of 25(OH)D_3 in COS-7 cells transfected with pcDNA3.1(−)-3(α→β)HSE. As shown in Fig. 7, the production of 3-epi-25(OH)D_3 from 25(OH)D_3 was not induced by transfection of 3(α→β)HSE. The conversion of ADT (3α) into epi-ADT (3β) and the reverse reaction, the transformation of epi-ADT (3β) into ADT (3α), were both detected by HPTLC. In addition, 5α-androstene-3,17-dione, which is the intermediate in the C-3 epimerization of ADT (3α) and epi-ADT (3β), was also detected (data not shown). These results suggest that the C-3 epimerization of 25(OH)D_3 was not catalyzed by CYP27A1, CYP27B1, CYP24 or 3(α→β)HSE.

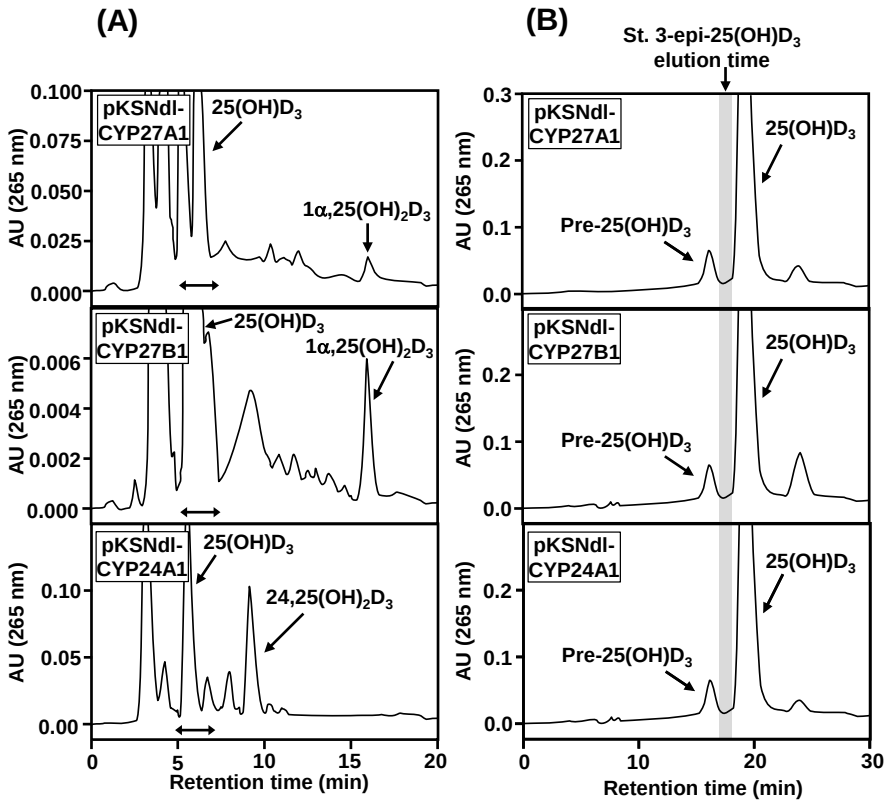

Fig. 6 Metabolism of 25(OH)D_3 in a reconstituted system containing the membrane fraction prepared from JM109/pKSNdl-CYP27A1, JM109/pKSNdl-CYP27B1 or JM109/pKSNdl-CYP24. (A) The first HPLC profile of the lipid extracts from the reaction mixture containing the membrane fraction prepared from JM109/pKSNdl-CYP27A1, JM109/pKSNdl-CYP27B1 and JM109/pKSNdl-CYP24. (B) The second HPLC profile of the fraction obtained from the first HPLC of the reaction mixture of CYP27A1, CYP27B1 and CYP24. The first HPLC analysis was performed using a Zorbax-SIL column (4.6 × 250 mm) eluted with hexane/2-propanol/methanol (HIM 88/10/2, v/v/v) at a flow rate of 1.0 mL/min. The second HPLC analysis was performed using a Sumichiral OA-2000 column (4.6 × 250 mm) eluted with 3.5 % 2-propanol/hexane at a flow rate of 1.0 mL/min.

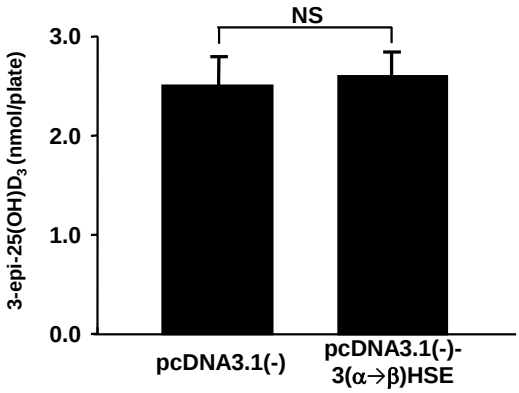

Fig. 7 Relative amounts of 3-epi-25(OH)D_3 generated from 25(OH)D_3 in COS-7 cells expressing 3(α→β)HSE. COS-7 cells transfected with pcDNA3.1(−) or pcDNA3.1(−)-3(α→β)HSE were incubated with 5 μM of 25(OH)D_3 for 48 h. The results are expressed as the total amount of 3-epi-25(OH)D_3 formed in nmol/plate/48h and represent the mean±SE of three experiments. There was no significant difference between the two groups. NS, not significantly different.

Discussion

The C-3 epimerization pathway leads to the conversion of the configuration of the hydroxyl group at C-3 of the A-ring and is quite different from side-chain oxidation pathways in view of the modification at the A-ring. Already known metabolic enzymes of vitamin D, such as CYP27A1, CYP27B1 and CYP24, belong to the cytochrome P450 family. In the present study, the microsomal C-3 epimerization activity was not inhibited by various cytochrome P450 inhibitors and antiserum against NADPH P450 reductase. We also confirmed that C-3 epimerization was not catalyzed by cytochrome P450 enzymes related to vitamin D metabolism, including CYP27A1, CYP27B1 and CYP24. Therefore, it is thought that the microsomal enzyme(s) responsible for the C-3 epimerization is not a member of the cytochrome P450 family. In UMR-106 cells, C-3 epimerization activity was not induced by pre-treatment with the active form of vitamin D, 1α,25$(OH)_2D_3$. Therefore, the expression of the enzyme(s) related to C-3 epimerization might not be regulated by 1α,25$(OH)_2D_3$. Steroid epimerase, 3(α→β)HSE, was one of the candidates for the enzyme which catalyzes the epimerization of the C-3 hydroxyl group in vitamin D compounds. It was reported that the activity of 3(α→β)HSE was observed in 100,000 × g fractions of cells transfected with 3(α→β)HSE, corresponding to the microsomal fraction. However, 3(α→β)HSE did not catalyze the C-3 epimerization of 25$(OH)D_3$ in this study. C-3 epimerization of steroids is composed of two steps, the oxidation of the C-3α hydroxyl group to the C-3 oxo group, followed by the reduction of the C-3 oxo group into the C-3β hydroxyl group. Higashi et. al. [27] speculated that 24,25$(OH)_2D_3$ was converted to 3-epi-24,25$(OH)_2D_3$ through the C-3 oxo intermediate from their experiments using 24,25$(OH)_2D_3$ labeled with deuterium at the C-3α position and identified 24,25-dihydroxy-9,10-secocholesta-4,7,10(19)-triene-3-one which was estimated to be a non-enzymatic

product derived from the C-3 oxo intermediate by treatment with cholesterol oxidase [34]. The C-3 oxo intermediates of vitamin D compounds are likely to be difficult to isolate since they are not stable. In our study using the microsomal fraction prepared from UMR-106 cells incubated with 25(OH)D_3, the peaks corresponding to the intermediates which have a C-3 oxo group were not detected in the predicted area of HPLC chromatograms [34].

In the present study, we optimized the conditions for the measurement of C-3 epimerization as follows: co-factors, a NADPH-generating system containing glucose-6-phosphate, NADP, glucose-6-phosphate dehydrogenase and Mg^{2+}; protein amount, 4 mg; incubation time, 60 min; pH, approximately 6.5; and buffer, potassium phosphate buffer. Then, we measured the C-3 epimerization activity in subcellular fractions prepared from UMR-106 cells, which metabolize vitamin D compounds to their C-3 epimers with relatively strong activity, and observed the highest level of activity in the microsomal fraction. In addition, microsomal fractions prepared from various cell lines also had C-3 epimerization activity toward 1α,25$(OH)_2D_3$. The activity for the reverse reaction from C-3α to C-3β was undetectable. These results suggest that enzyme(s) responsible for the epimerization of vitamin D at C-3 might be localized to microsomes.

It was reported that both 3α-HSD and β-HSD purified from bacteria (*Pseudomonas testosterone*) catalyzed C-3 epimerization from 24,25$(OH)_2D_3$ (3β) to 3-epi-24,25$(OH)_2D_3$ (3α) in the presence of NAD and NADPH [34]. 3α-HSD and β-HSD also catalyzed the reverse reaction from 3-epi-24,25$(OH)_2D_3$ (3α) to 24,25$(OH)_2D_3$ (3β). These findings disagree with the unidirectional reaction from C-3β to C-3α which was observed in mammalian cells [25, 26, 29]. Moreover, the activity of 3α-HSD and β-HSD has been reported to be localized in cytosol [35–37]. Thus, 3α-HSD and β-HSD would contribute little to C-3 epimerization activity observed in microsome.

The kinetic parameters for the C-3 epimerization were also calculated using a microsomal fraction prepared from UMR-106 cells. The highest Vmax/Km value was for 25(OH)D_3 among all the substrates tested indicating that 25(OH)D_3 is a good the substrate for C-3 epimerization. In contrast, the lower Vmax/Km values for 24,25$(OH)_2D_3$ and OCT than for 25(OH)D_3 and 1α,25$(OH)_2D_3$ suggested that the introduction of a polar group into the side-chain reduces the C-3 epimerization activity. These results are consistent with our previous finding that 24,25$(OH)_2D_3$ and OCT were little metabolized to their C-3 epimers in various cultured cells [26, 28, 29].

The epimerization of other functional molecules has been shown to have important roles. One well-known epimerase is uridine diphosphate (UDP)-glucose 4-epimerase (EC 5.1.3.2), which catalyzes the conversion of UDP-glucose into UDP-galactose and has been associated with a disease called galactosemia [39–42]. In addition, the importance of epimerization in regulating the biological activities of steroid hormones has been demonstrated. The neuroactive steroid, 5α-pregnane-3α-ol-20-one, plays a role in the modulation of reproductive function by suppressing the release of hypothalamic gonadotropin-releasing hormone [43]. However, its C-3 epimer, 5α-pregnane-3β-ol-20-one, is ineffective in regulating hypothalamic activity [44]. The significance of the C-3 epimerization pathway of vitamin D is not fully understood. The relative VDR-binding affinity, transcriptional activity, and anti-proliferative/differentiation-inducing activity of C-3 epimers (C-3α) are weaker than those of C-3β compounds as described in Part 2, Chapter 1. It was also noted that the C-3 epimerization pathway is cell-selective [25, 26, 29] and 3-epi-1α,25$(OH)_2D_3$ is almost equipotent to 1α,25$(OH)_2D_3$ in suppressing parathyroid hormone secretion in bovine parathyroid cells [45] and in inhibiting keratinocyte proliferation [46]. In addition, 3-epi-1α,25$(OH)_2D_3$ is more potent than

$1\alpha,25(OH)_2D_3$ in inducing apoptosis in human promyelocytic leukemia (HL-60) cells [47]. These results suggest that the C-3 epimerization pathway might plays an important role in the formation of metabolites with a different profile of biological activity. Further studies including identification of the enzyme(s) which can catalyze the epimerization of the hydroxyl group at C-3 will be needed to elucidate of the biological significance of the C-3 epimerization pathway of vitamin D.

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GENERAL CONCLUSION

In this study, we measured vitamin D levels in biological samples using LC-APCI/MS/MS and CLIA techniques. We also identified several novel metabolites of vitamin D and examined their biological activity.

In Part 1, sensitive quantification method for vitamin D and other fat-soluble vitamins in human breast milk by LC-APCI/MS/MS was developed. For the determination of vitamin D compounds, derivatization with a Cookson-type reagent was performed to improve sensitivity. The mean concentration of D_3, D_2, 25(OH)D_3 and 25(OH)D_2 were 0.088, 0.078, 0.081 and 0.003 ng/mL, respectively (n=82). Daily intake of vitamin D calculated from an infant's consumption of breast milk, 780 mL/day and Reeve's conversion factor was approximately 0.5 μg, which did not meet current DRIs (AI, 5.0 μg/day). These results suggest that supplementation of vitamin D for breast feeding mothers or breastfed infants is beneficial in improving the vitamin D status of breast fed infants. We also measured serum 25(OH)D concentration in over 1300 healthy adolescents aged 12-18 years by CLIA. The mean serum 25(OH)D concentrations in boys and girls were 60.8 and 52.8 nmol/L (24.3 and 21.1 ng/mL), respectively. Approximately 30 % of boys and 48 % of girls had sub-optimal 25(OH)D concentrations (50 nmol/L, 20 ng/mL). Serum PTH concentration was negatively correlated with serum 25(OH)D concentrations in boys, but negatively correlated with calcium intake rather than serum 25(OH)D in girls. In contrast, the increment in calcaneal stiffness as a result of elevation of serum 25(OH)D was higher in girls than in boys. Thus, improvement of vitamin D and calcium status, especially in girls, should be importance for bone health in Japanese adolescents.

In Part 2, first, the isolation and structural assignment of 3-epi-25(OH)D_3 as a major metabolite of 25(OH)D_3 and further metabolism of C-3 epimers of vitamin D are described.

3-epi-25(OH)D_3 was generated from 25(OH)D_3 in various cultured cells *in vitro* including osteosarcoma, colon adenocarcinoma and hepatoblastoma cell line, and rats *in vivo*. In cultured cells, 3-epi-25(OH)D_3 and 3-epi-1α,25$(OH)_2D_3$ were metabolized to 3-epi-24,25$(OH)_2D_3$ and 3-epi-1α,24,25$(OH)_3D_3$, respectively. In addition, we demonstrated that 3-epi-25(OH)D_3 was metabolized to 3-epi-1α,25$(OH)_2D_3$ by CYP27B1 and to 3-epi-24,25$(OH)_2D_3$ by CYP24A1 using recombinant *E. Coli* cell systems. 3-Epi-25(OH)D_3, 3-epi-1α,25$(OH)_2D_3$ and 3-epi-24,25$(OH)_2D_3$ were biologically less active than 25(OH)D_3, 1α,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$, but 3-epi-1α,25$(OH)_2D_3$ showed to some extent transcriptional activity toward target genes and anti-proliferative/differentiation-inducing activity. These results indicate that C-3 epimerization may be a common metabolic pathway for the major metabolites of D_3. Next, *in vitro* metabolism of OCT was studied to identify the less polar metabolites and to assess their biological activity. OCT was metabolized to three less polar metabolites, 3-epi-OCT and two dehydrates, 25-ene-22-oxa-1α(OH)D_3 and 24-ene-22-oxa-1α(OH)D_3. We also observed further metabolites, the two C-25 dehydrates of 3-epi-OCT. The biological activity including VDR binding affinity, DBP binding affinity and cell differentiation activity was found to be lower than OCT. Thus, both the C-3 epimerization and C-25 dehydration may work to reduce the biological activity of OCY. Finally, we optimized the conditions for the measurement of C-3 epimerization activity. The highest level of activity was observed in microsomal fraction prepared from various kind of cells. Judging from Vmax/Km, 25(OH)D_3 exhibited the highest specificity for the epimerization in compounds tested. Neither CYP24A1, CYP27A1, CYP27B1 nor 3(α→β)HSE catalyzed the epimerization. Based on these results, the enzyme(s) responsible for C-3 epimerization of vitamin D are thought to be located in microsomes and different from the known metabolic enzymes of vitamin D and 3(α→β)HSE.

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Part 1 Chapter 1

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Part 1 Chapter 2

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Part 2 Chapter 1

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Part 2 Chapter 2

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論文内容の要旨

Studies on Vitamin D Levels in Biological Samples and Novel Metabolites of Vitamin D

「生体試料中ビタミン D 濃度と新規ビタミン D 代謝物に関する研究」

鎌尾　まや

はじめに

ビタミンDはカルシウム恒常性や骨代謝に重要な栄養素であることはよく知られているが、近年、免疫系や生殖、がん予防など、カルシウム代謝調節以外の生理作用についても関心が集まっている。ビタミン D_3（D_3）は皮膚において 7-dehydrocholesterol の紫外線照射により生成し、肝臓で 25-hydroxyvitamin D_3 [25(OH)D_3] に代謝された後、腎臓で活性型である 1α,25-dihydroxyvitamin D_3 [1α,25(OH)$_2D_3$] あるいは不活性型である 24,25-dihydroxyvitamin D_3 [24,25(OH)$_2D_3$] に代謝される。これらのうち 25(OH)D_3 は血中に最も多く存在するビタミン D 代謝物であり、D_3 の紫外線照射による生成量や食事からの摂取量を最もよく反映する栄養学的な指標となる。ビタミン D_2（D_2）はきのこ類に多く含まれる植物性のビタミンDであるが、D_3 と同様に 25 位が水酸化された後、1α位あるいは 24 位が水酸化され、活性化あるいは不活性化される。現在までにこれら以外にも多くの代謝物の構造が決定されているが、さらに多くの未同定代謝物も存在するものと考えられている。

近年の疫学研究において、血清（あるいは血漿）中 25-hydroxyvitamin D [25(OH)D, 25(OH)D_3 と 25(OH)D_2 の総量を示す] 濃度が低値であることが、骨折に加え、アルツハイマー、２型糖尿病および新生児の急性上気道感染などのリスクを高めることが報告されている。これらの結果は、乳児から老年期に至る全てのライフステージでビタミン D が健康維持や疾患予防に重要な栄養素であることを示唆している。従って、血清や母乳などの生体試料中におけるビタミン D 代謝物濃度を測定し、その標準的な値を把握することは極めて有用である。また、多岐にわたるビタミンDの生理作用やその調節機構を明らかにする上で、ビタミン D の未知代謝物を同定しその生理活性を明らかにすることは重要である。これらの背景から、本研究では母乳および血清中ビタミン D 代謝物濃度を測定し、関連因子との相関性を解析した。また、培養細胞を用いて天然型ビタミン D および合成誘導体の新規代謝物を同定し、その生理活性を評価すると共に、新規代謝物産生活性の測定系を構築した。

第１部　生体試料中ビタミンD濃度の分析

第１章　母乳中ビタミンDおよび他の脂溶性ビタミンの定量

母乳中に含まれるビタミンD量は少ないため、専ら母乳で哺育され、かつ日照不足の乳児はビタミン D 欠乏に陥るリスクが高い。また、その含有量の少なさから、母乳中ビタミン D を正確に定量することは困難であった。筆者らは、液体クロマトグラフタンデム型質量分析計を用いた高感度で特異性に優れた脂溶性ビタミンの定量法を確立し、比較的少量の母乳よりビタミンDおよび他の脂溶性ビタミン含量を測定することに成功した。そこで、日本人授乳婦（82 名、18－39 歳）を対象として母乳中ビタミン D 含量を調査したところ、平均濃度は D_3 0.088 ng/mL、D_2 0.078 ng/mL、25(OH)D_3 0.081 ng/mL、25(OH)D_2 0.003 ng/mL といずれも低値であった。また、他の脂溶性ビタミン濃度は、レチノール 0.455 μg/mL、β-カロテン 0.062 μg/mL、α-トコフェロール 5.087 μg/mL、フィロキノン 3.771 ng/mL、メナキノン-4　1.795 ng/mL、メナキノン-7　1.540 ng/mL であった。母乳中ビタミン D 濃度より Reeve の換算係数 [25(OH)D は D の５倍とする] を用いて総ビタミン濃度を算出し、乳児の平均哺乳量 780 mL/day を乗じて乳児のビタミンD摂取量を推定したところ約 0.5 μg/day であり、本邦の食事摂取基準における目安量 5 μg/day に比べて極めて低い値であった。従って、日照不足が懸念される冬季には特に、授乳婦や乳児を対象としたビタミン D 補充が必要であると考えられた。

第２章　日本人の思春期男女を対象とした血清中 25(OH)D の測定

超高齢社会を迎えた我が国では骨粗鬆症の増加が懸念されており、その予防として思春期における最大骨量を高めることが重要である。思春期は骨代謝の変化が大きく、ビタミン D 栄養が極めて重要な時期であるが、思春期の日本人を対象とした研究は乏しいのが現状

である。そこで筆者らは、日本人の健常思春期男女 1,380 名（男子 662 名、女子 718 名、12－18 歳）を対象として血清中 25(OH)D 濃度を化学発光免疫測定法（CLIA）で自動測定した。その結果、血清中 25(OH)D 濃度の平均値は男子で 60.8 nmol/L（24.3 ng/mL）、女子で 52.8 nnol/L（21.1 ng/mL）となり、女子の方が有意に低いことが明らかになった。また、血清中 25(OH)D 濃度は男子では 30%、女子では 48%が 50 nmol/L（20 ng/mL）以下であり、多くの対象者がビタミン D 不足境界領域であった。諸外国において血清中 25(OH)D 濃度と副甲状腺ホルモン（PTH）濃度が逆相関を示すと報告されている。本集団において 25(OH)D 濃度と PTH 濃度は全学年の男子で逆相関を示したが、女子では逆相関を示したのは高校 3 年生のみであった。一方、男女共に踵骨骨密度は血清中 25(OH)D 濃度と有意な正相関を示したが、その相関性は女子の方が強かった。また、男女共にカルシウム摂取より血清中 25(OH)D 濃度が踵骨骨密度に強く影響を及ぼしたが、その傾向は女子の方が強かった。以上より、男女共に思春期における骨密度増加にはカルシウムに加え、ビタミン D のサプリメントが有益であると判断された。

第２部　ビタミン D の新規代謝物の同定

第１章　天然型ビタミン D の 3 位水酸基異性体の同定

天然型ビタミン D 代謝物のうち、1α,25(OH)$_2$D$_3$ および 24,25(OH)$_2$D$_3$ は 3 位の水酸基がβ位からα位に異性化した代謝物に代謝されることが *in vitro* および *in vivo* で明らかにされている。この 3 位水酸基の異性化は代謝部位が A 環部である点が 24 位あるいは 23 位水酸化経路とは異なっており、多様な代謝物の産生という観点から大変興味深い代謝経路である。そこで筆者らは、生体内で最も多く存在している 25(OH)D$_3$ を基質として、その 3 位水酸基異性体 3-epi-25(OH)D$_3$ が培養細胞により産生されるかを検討した。その結果、用いた 5 種類の細胞全てにおいて 3-epi-25(OH)D$_3$ に相当する代謝物の産生が確認され、精製画分の NMR および LC-MS 解析により 25(OH)D$_3$ の 3 位水酸基異性体であると同定した。3-epi-25(OH)D$_3$ は骨、腸あるいは肝臓由来の細胞で主代謝物であったが、腎臓由来の細胞では 24,25(OH)$_2$D$_3$ が主代謝物であった。25(OH)D$_3$ からの 3 位水酸基異性体の産生量は、1α,25(OH)$_2$D$_3$ や 24,25(OH)$_2$D$_3$ を基質とした場合に比べて多く、細胞内で 25(OH)D$_3$ は他の天然型ビタミン D 代謝物に比べて 3 位水酸基異性体に代謝されやすいと判断された。続いて、培養細胞における 3-epi-25(OH)D$_3$ および 3-epi-1α,25(OH)$_2$D$_3$ の代謝を検討したところ、それぞれの 24 位水酸化体が生成したが、25(OH)D$_3$ あるいは 1α,25(OH)$_2$D$_3$ の生成は認められなかった。さらに、ビタミン D の 1α位水酸化酵素（CYP27B1）あるいは 24 位水酸化酵素（CYP24A1）の大腸菌発現系を用いて、3-epi-25(OH)D$_3$ が 25(OH)D$_3$ と同様に、CYP27B1 により 3-epi-1α,25(OH)$_2$D$_3$ に、CYP24A1 により 3-epi-24,25(OH)$_2$D$_3$ に代謝されることを明らかにした。3-epi-25(OH)D$_3$、3-epi-1α,25(OH)$_2$D$_3$ および 3-epi-24,25(OH)$_2$D$_3$ の VDR 結合能をはじめとする生理活性はそれぞれの 3β体に比べて弱かったが、3-epi-1α,25(OH)$_2$D$_3$ は 1α,25(OH)$_2$D$_3$ の 30％程度のヒト前骨髄性白血病細胞増殖抑制能を保持していた。以上より、3 位水酸基の異性化はビタミン D 代謝物に普遍的な代謝経路であると判断された（図１）。

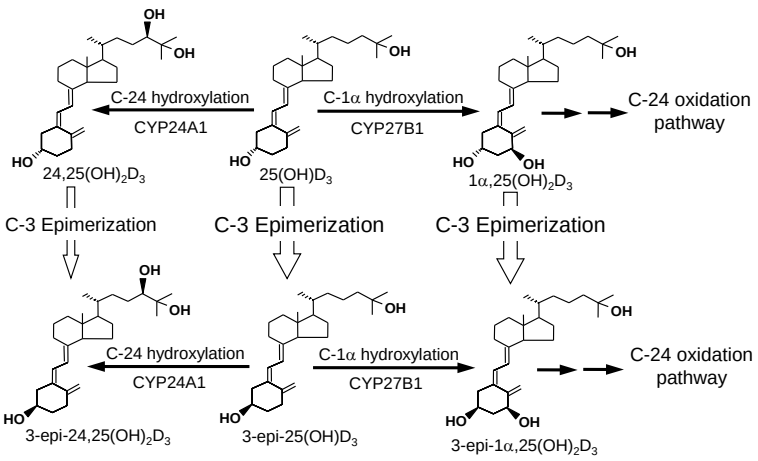

図１．天然型ビタミン D 代謝物の 3 位水酸基異性化による代謝

第２章　ビタミン D 誘導体 22-oxa-calcitriol（OCT、maxacalcitriol）の 3 位水酸基異性体および 25 位脱水体の同定

OCT は 1α,25(OH)$_2$D$_3$ の 22 位の炭素原子を酸素原子に置換した合成誘導体であり、カルシウム作用が弱く細胞の増殖抑制作用や分化誘導能が強い特徴を有している。日本において OCT は二次性副甲状腺機能亢進症や乾癬の治療薬として臨床応用されており、その代謝を詳細に検討することは重要である。筆者らは、培養細胞により OCT からいくつかの低極性代謝物が産生されることに着目し、NMR および LC-MS 解析により構造を決定した。OCT から産生した 3 種類の低極性代謝物のうち一つは 3 位水酸基異性体であり、他の二つは側鎖の 25 位の水酸基の脱水により産生する 25-ene-22-oxa-1α(OH)D$_3$ および 24-ene-22-oxa-1α(OH)D$_3$ であった（図２）。骨、腸および腎臓由来の細胞でこれら３種の代謝物の生成量を比較したところ、各代謝物の生成比は細胞により全く異なっており、骨由来細胞では 25-ene 体と 24-ene 体の生成比は 2：1 であったのに対し、腸および腎臓由来の細胞では 1：4 および 1 : 11 であった。さらに、3-epi-OCT は 25 位水酸基の脱水体 25-ene-3-epi-22-oxa-1α(OH)D$_3$ および 24-ene-3-epi-22-oxa-1α(OH)D$_3$ に代謝された。3-epi-OCT、25-ene-22-oxa-1α(OH)D$_3$ および 24-ene-22-oxa-1α(OH)D$_3$ の生物活性はいずれも極めて弱く、OCT において 3 位水酸基の異性化あるいは 25 位水酸基の脱水による代謝は、標的細胞内での活性を弱める役割を担うと考えられた。

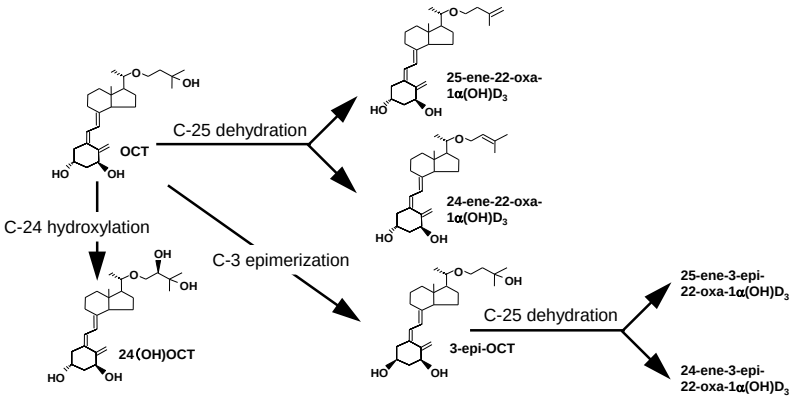

図２．OCT の 3 位水酸基異性化および 25 位水酸基脱水による代謝

第３章　３位水酸基異性化活性の測定

第２部第１章、第２章により、天然型ビタミン D 代謝物や合成誘導体が 3 位水酸基の異性化により代謝されることが明らかとなった。筆者らは、ビタミン D の 3 位水酸基異性化反応を触媒する酵素の基本的性質を明らかにする目的で、まず、活性測定法の至適条件を確立した。その条件を用いて、各細胞画分における 3 位水酸基異性化活性を測定したところ、ミクロソーム画分で最も高い活性が認められた。また、UMR-106 細胞のミクロソーム画分を酵素源とし、25(OH)D$_3$、1α,25(OH)$_2$D$_3$、24,25(OH)$_2$D$_3$ および OCT を基質として Vmax/Km を比較したところ、25(OH)D$_3$ が最も大きく、良い基質になると判断された。さらに各種シトクロム P450 酵素阻害剤および NADPH P450 レダクターゼ抗血清による阻害試験をおこなったが、いずれにおいても 3 位水酸基異性化活性の有意な阻害は認められなかった。また、既知の 3 種のビタミン D 代謝酵素（CYP27A1、CYP27B1 および CYP24A1）を大腸菌に発現させて得られた膜タンパクおよびステロイドの 3 位水酸基異性化酵素である 3(α→β)hydroxysteroid epimerase（HSE）を過剰発現させた培養細胞を用いて 25(OH)D$_3$ からの 3-epi-25(OH)D$_3$ 産生量を比較したが、いずれも有意な変化はみられなかった。よって、ビタミン D の 3 位水酸基異性化反応に関与する酵素は既知のビタミン D 代謝酵素などのシトクロム P450 系の酵素や HSE ではないと判断された。