

Vitamin D Metabolism and Action in Human Bone Marrow Stromal Cells

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Vitamin D metabolites are important effectors of bone and mineral homeostasis. Extrarenal conversion of 25-hydroxyvitamin D (25OHD) to the biologically active form of vitamin D, 1,25-dihydroxyvitamin D [$1,25(\text{OH})_2\text{D}$] is catalyzed in several cell types by the 1α -hydroxylase (CYP27B1), but little is known about the expression or regulation of CYP27B1 in human bones. We examined whether human bone marrow stromal cells (hMSCs, also known as mesenchymal stem cells) participate in vitamin D metabolism and whether vitamin D hydroxylases in hMSCs are influenced by the vitamin D status of the individual from whom the hMSCs were obtained. We also investigated the effects of vitamin D metabolites on osteoblast differentiation and the role of IGF-I in the regulation of CYP27B1. In a series of 27 subjects, vitamin D hydroxylases in hMSCs were expressed at different levels and were correlated with serum 25OHD, $1,25(\text{OH})_2\text{D}$, and PTH. *In vitro* treatment with 25OHD up-regulated CYP27B1 and IGF-I in hMSCs; IGF-I also up-regulated CYP27B1 expression and stimulated osteoblast differentiation. When hydroxylation of 25OHD was blocked by ketoconazole, a cytochrome P450 inhibitor, 25OHD was no longer able to induce CYP27B1 expression. In summary, these findings show that human bone marrow stromal cells have the molecular machinery both to metabolize and respond to vitamin D. We propose that circulating 25OHD, by virtue of its local conversion to $1,25(\text{OH})_2\text{D}$ catalyzed by basal CYP27B1 in hMSCs, amplifies vitamin D signaling through IGF-I up-regulation, which in turn induces CYP27B1 in a feed-forward mechanism to potentiate osteoblast differentiation initiated by IGF-I. (*Endocrinology* 151: 14–22, 2010)

There are two major sources of vitamin D (cholecalciferol): dietary intake and conversion of 7-dehydrocholesterol in the skin to vitamin D by UV light. Vitamin D from both sources undergoes sequential steps of activation, a first hydroxylation in the liver to 25-hydroxyvitamin D (25OHD) and a second hydroxylation in the kidney to the active hormone 1,25-dihydroxyvitamin D [$1,25(\text{OH})_2\text{D}$] (1). The active metabolite, $1,25(\text{OH})_2\text{D}$, is taken up by target cells that possess the vitamin D receptor (VDR). Vitamin D status is assessed by the circulating level of 25OHD. Vitamin D deficiency can lead to low bone density and, in severe instances, osteomalacia (2) and is associated with osteopenia and osteoporosis, muscle weakness, falls, and increased risk of fracture (3–5). We

reported extreme vitamin D deficiency in U.S. women with hip fractures (6). Emerging evidence indicates that vitamin D deficiency is associated with many nonskeletal illnesses, including cancer, autoimmune diseases, infectious diseases, and cardiovascular disease (7).

Among the cytochrome P450 (CYP) isoforms that have been shown to hydroxylate vitamin D, CYP27B1/25OHD- 1α -hydroxylase converts 25OHD into the active hormonal form, $1,25(\text{OH})_2\text{D}$. CYP27B1 activity is tightly regulated through complex mechanisms that depend on the circulating levels of calcium, phosphorus, PTH, and $1,25(\text{OH})_2\text{D}_3$ (8) and by calcitonin (9). Previous studies suggest that IGF-I may regulate the renal production of $1,25(\text{OH})_2\text{D}_3$ (10–14). In addition to kidney tubule cells,

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Abbreviations: AlkP, Alkaline phosphatase; BMD, bone mineral density; BMI, body mass index; CYP, cytochrome P450; FBS-HI, fetal bovine serum-heat inactivated; hMSC, human bone marrow stromal cell; NTX, cross-linked N-telopeptides of type I collagen; 25OHD, 25-hydroxyvitamin D; $1,25(\text{OH})_2\text{D}$, 1,25-dihydroxyvitamin D; VDR, vitamin D receptor.

other human cells have been demonstrated to produce $1,25(\text{OH})_2\text{D}$, notably osteoblasts (15), activated macrophages (16), keratinocytes (17), endothelial cells (18), and cancer cells (19). Finding the VDR and vitamin D hydroxylases in many tissues suggests that the vitamin D hormone acts in an autocrine, paracrine, or intracrine fashion to affect the biology of nonclassical target tissues. Recent data from mouse studies appear to show more limited distribution of 1α -hydroxylase (8).

Human marrow-derived stromal cells (hMSCs), also known as mesenchymal stem cells, include progenitors of several lineages, including osteoblasts, chondrocytes, and adipocytes (20–22). From studies with hMSCs isolated from marrow that was discarded during orthopedic surgery, we determined that there is an age-related decrease in their differentiation to osteoblasts (23, 24). The differentiation of hMSCs to osteoblasts is enhanced by $1,25(\text{OH})_2\text{D}_3$ (25), but there is no information about vitamin D metabolism in hMSCs or the effects of 25OHD on these processes. In this series of investigations, we tested whether 25OHD_3 stimulates hMSCs to differentiate to osteoblasts, whether hMSCs participate in vitamin D metabolism, whether expression of vitamin D hydroxylases in hMSCs *in vitro* are influenced by the vitamin D status of the individual from whom the hMSCs were obtained, and whether vitamin D metabolic enzymes in hMSCs are regulated *in vitro*.

Materials and Methods

Subjects

Bone marrow samples were obtained with Institutional Review Board approval and annual review as femoral tissue discarded during primary hip arthroplasty for osteoarthritis. Criteria for exclusion were rheumatoid arthritis, cancer, and other comorbid conditions that may influence skeletal metabolism, *i.e.* renal insufficiency, alcoholism, active liver disease, malabsorption, hyperthyroidism, ankylosing spondylitis, aseptic necrosis, hyperparathyroidism, morbid obesity, and diabetes. Also excluded were patients who were taking medications that may influence skeletal metabolism (*e.g.* thyroid hormone, glucocorticoids, bisphosphonates, and nonsteroidal antiinflammatory drugs). A set of 27 subjects scheduled for hip arthroplasty was consented for research studies, including measurement of serum 25OHD , $1,25(\text{OH})_2\text{D}$, and PTH as well as body composition and bone mineral density (BMD). Although current estrogen use was not excluded in the screening criteria, none of the women in this series was receiving estrogen at the time of surgery. Another set of bone marrow samples that was used for osteoblast differentiation experiments was obtained as discarded tissue from 19 de-identified individuals with Institutional Review Board approval and the same preoperative exclusion screen.

Blood chemical assays

Blood chemistries and complete blood counts were performed in hospital clinical laboratories; the remaining tests were

performed in the General Clinical Research Center laboratory unless otherwise specified. Serum 25OHD levels were assayed using an isotopic assay (DiaSorin RIA, Stillwater, MN), with a sensitivity of 1.5 ng/ml and an interassay coefficient of variation (CV) of less than 10.5%; sufficiency was defined as more than 32 ng/ml (26). Levels of $1,25(\text{OH})_2\text{D}$ were measured by an extraction and isotopic method (DiaSorin) with a sensitivity of 2 pg/ml and an interassay CV of less than 14.7%; the normal range was 15–75 pg/ml. Serum intact PTH levels were measured with the sensitive chemiluminescent assay (Beckman Access II; Beckman Coulter, Inc., Fullerton, CA), with a sensitivity of 1 pg/ml and an interassay CV of less than 6.5%; the normal range of serum PTH was 10–65 pg/ml. Urinary N-telopeptide levels corrected for urinary creatinine, an index of bone resorption, were determined in a second morning spot urine collection by an ELISA that measures cross-linked collagen peptides (Osteomark Assay; Ostex International, Inc., Seattle, WA), the normal range was 13–65 nmol/mmol creatinine.

BMD and body composition

BMD of the spine (L1–L4) and proximal femur were measured with dual x-ray absorptiometry technique (Discovery; Hologic Inc., Bedford, MA). Least significant changes at the 95% confidence level for the spine and femoral neck bone density measurements were 0.017 and 0.014 g/cm², respectively. Vertebrae with moderately severe osteoarthritic changes, disc space narrowing, or a fracture would be excluded from the analyses as those anatomic findings may elevate the spinal BMD. For these 27 subjects, none of the spine BMD measurements had to be excluded. If subjects had a hip replacement on the contralateral side, BMD was not measured at that site. Results were expressed as SD compared with BMD values for young normal controls (T-score). Body composition was also determined by dual x-ray absorptiometry (Discovery; Hologic) (27). Reproducibility for fat determination in our laboratory was $1.09 \pm 0.15\%$ (CV, mean \pm SEM).

Preparation of hMSCs

Low-density marrow mononuclear cells were isolated by centrifugation on Ficoll/Histopaque 1077 (Sigma Chemical Co., St. Louis, MO) (28). This procedure removes differentiated cells and enriches for undifferentiated, low-density marrow mononuclear cells that include a population of nonadherent hematopoietic cells and a fraction capable of adherence and differentiation into musculoskeletal cells. Adherent hMSCs were expanded in monolayer culture with phenol red-free α -MEM, 10% fetal bovine serum-heat inactivated (FBS-HI), 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (Invitrogen, Carlsbad, CA). All samples were used at passage 2.

Alkaline phosphatase (AlkP) enzyme assay

A set of MSCs that were obtained from 19 de-identified subjects was used to assess osteoblast differentiation *in vitro*. Cells were cultured in triplicate in 12-well plates in α -MEM with 10% FBS-HI until confluence; this required different times depending upon rates of proliferation. Upon confluence, medium was changed to osteogenic medium (α -MEM with 1% FBS-HI, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin plus 10 nM dexamethasone, 5 mM β -glycerophosphate, and 50 $\mu\text{g}/\text{ml}$ ascorbate-2-phosphate) for 7 d. Reduction of serum to 1% for differentiation was designed to minimize possible differences in proliferation

that could confound interpretation of effects of vitamin D₃ metabolites or other agents on osteoblastogenesis. AlkP enzyme activity was measured as previously described (24). Cells from an enrolled 72-yr-old woman were used to measure the effect of IGF-I on osteoblastogenesis.

RNA isolation and RT-PCR

Total RNA was isolated from hMSCs with Trizol reagent (Invitrogen). For RT-PCR, 2 μg total RNA was reverse-transcribed into cDNA with SuperScript II (Invitrogen), following the manufacturer’s instructions. One twentieth of the cDNA was used in each 50-μl PCR (30–40 cycles of 94 C for 1 min, 55–60 C for 1 min, and 72 C for 2 min) as described (28). The gene-specific primers for human CYP27B1 (29), CYP24A1 (30), VDR (31), and IGF-I (32) were used for amplification. CYP27A1/25O₃Hase primers were modified from Seifert et al. (30): forward 5'-GGAAAGTACCCAGTACGG-3' and reverse 5'-AGCAAAT-AGCTTCCAAGG-3' (289-bp product). Concentration of cDNA and amplification conditions were optimized to reflect the exponential phase of amplification. Quantitative data were expressed by normalizing the densitometric units to GAPDH (internal control) as described (24, 28).

In vitro biosynthesis of 1,25(OH)₂D₃ by hMSCs

hMSCs were cultivated in 12-well plates until confluence and then treated with or without 1 μM 25OHD₃ (Sigma) with or without 10 μM CYP inhibitor ketoconazole (Sigma) or 100 ng/ml IGF-I (R&D Systems, Minneapolis, MN) in serum-free α-MEM supplemented with 1% ITS⁺1 (Sigma). 1,2-Dianilinoethane (N,N'-diphenylethylene-diamine) (10 μM) (Sigma) was added into the cultures as an antioxidant as described (18). After 24 h treatment, the media were collected from each well. The 1,25(OH)₂D₃ levels in media were quantitatively determined with a 1,25(OH)₂D₃ ELISA kit (USA Immunodiagnostic Systems Ltd., Fountain Hills, AZ), according to the manufacturer’s instructions. The hMSCs were lysed with a buffer containing 150 mM NaCl, 3 mM NaHCO₃, 0.1% Triton X-100, and a mixture of protease inhibitors (Roche Diagnostics, Pleasanton, CA). Protein concentration was determined with the BCA system (Pierce, Rockford, IL). The 1α-hydroxylase activity was expressed as biosynthesized 1,25(OH)₂D₃ in medium per milligram protein per hour of 25OHD₃ treatment (femtomoles per milligram protein per hour).

Statistical analyses

All experiments were performed at least in triplicate. Group data are presented as mean values ± SD. Unless otherwise indicated, quantitative data were analyzed with nonparametric tools, either the Mann-Whitney U test for group comparisons or Spearman correlation test. If data allowed, parametric tools were used, either t test for two-group or one-way ANOVA for multiple-group comparisons or Pearson correlation test. A value of P < 0.05 was considered significant.

Results

In vitro stimulation of osteoblast differentiation by both 25OHD₃ and 1,25(OH)₂D₃

A set of MSCs that were obtained from 19 de-identified subjects was used to assess osteoblast differentiation in

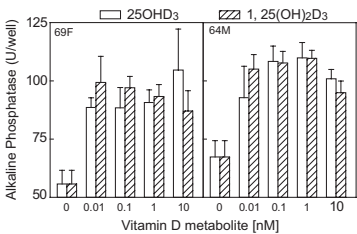


FIG. 1. Effects of vitamin D metabolites on osteoblast differentiation in hMSCs from a 69-yr-old female (69F) and a 64-yr-old male (64M) subject. Osteoblast differentiation of hMSCs was assessed with AlkP enzyme activity assays (n = 6) after 7 d culture with or without 25OHD₃ or 1,25(OH)₂D₃ (0.01–10 nM) in osteogenic medium.

vitro. There were samples from 10 men and nine women between 64 and 83 yr of age. Cultures of these hMSCs were treated with either 1,25(OH)₂D₃ or 25OHD₃ (0.01–10 nM) for 7 d in osteogenic medium. Osteoblast differentiation, assessed with AlkP enzyme activity assays, was stimulated by 1,25(OH)₂D₃ in all but two of the 19 samples (89%), with peak stimulation between 1 and 10 nM. Two thirds of the samples were stimulated by both 1,25(OH)₂D₃ and 25OHD₃. In some cases, there was equivalent dose-response stimulation of osteoblast differentiation in hMSCs by both 1,25(OH)₂D₃ and 25OHD₃ (Fig. 1). In those examples, both 1,25(OH)₂D₃ and 25OHD₃ (0.01–10 nM) significantly stimulated AlkP activity, compared with vehicle controls (P < 0.001), and there was no significant difference between 1,25(OH)₂D₃ and 25OHD₃.

Characteristics of the study subjects

Clinical data were available for 27 consented subjects, from whom MSCs were isolated from bone marrow discarded during orthopedic surgery. The mean age was 66 ± 10 yr, ranging from 41–81 yr. There were 14 men and 13 women. There was a wide range of values for serum 25OHD, 1,25(OH)₂D, PTH, cross-linked N-telopeptides of type I collagen (NTX), urine creatinine levels, percent fat, body mass index (BMI), and BMD T-scores (Table 1). We found that 28% of the subjects were vitamin D defi-

TABLE 1. Characteristics of the study subjects (n = 27, except as noted)

	Mean ± SD	Range
Age (yr)	66 ± 10	41–81
25OHD (ng/ml)	27.5 ± 10.8	7.6–48.8
1,25(OH) ₂ D (pg/ml) (n = 23)	40.3 ± 12.5	20.0–71.0
PTH (pg/ml) (n = 24)	37.6 ± 16.6	12.1–83.7
Urine creatinine (mg/dl)	97.7 ± 47.6	22.5–213.3
Urine NTX (nmol/mmol creatinine) (n = 25)	43.6 ± 18.8	14.0–84.0
Spine T-score	0.54 ± 1.74	–2.8 to 3.7
Left total hip T-score (n = 22)	–0.45 ± 1.12	–1.9 to 1.9
BMI (kg/m ²) (n = 26)	29.1 ± 6.6	19.9–49.3
% Fat	35.6 ± 8.9	19.1–49.1

TABLE 2. Correlations between serum 25OHD and other clinical parameters

	Serum PTH	Serum 1,25(OH) ₂ D	BMI	% Fat
r	−0.49	0.016	−0.41	−0.50
n	24	23	26	27
P	0.015	0.94	0.035	0.0077

n, Number of subjects; r, Spearman correlation coefficient.

cient (<20 ng/ml serum 25OHD), that 36% were insufficient with level between 20 and 32 ng/ml, and that 36% were vitamin D sufficient (>32 ng/ml). There were no differences in serum 25OHD between genders or with age. Serum 25OHD was inversely correlated with serum PTH ($r = -0.49$; $P = 0.015$), with BMI ($r = -0.41$; $P = 0.035$), and with percent body fat (Spearman $r = -0.50$; $P = 0.0077$) (Table 2). There were no correlations with 1,25(OH)₂D, urine NTX, or T-score of spine and total hip. There was a trend for an inverse correlation between serum 25OHD₃ and urinary creatinine ($r = -0.43$; $P = 0.058$).

Relationships between serum parameters and constitutive expression of vitamin D hydroxylases in hMSCs

Gene expression was assessed in hMSCs from 27 subjects for whom clinical information was available. In this series, there was a wide range of constitutive expression of 1 α -hydroxylase (CYP27B1), with no differences between genders or with age (for clarity, 17 representative samples are shown in Fig. 2A). All 27 samples expressed the VDR, IGF-I, and CYP27A1/25-hydroxylase, whereas 18 of 27 had detectable CYP24A1/24-hydroxylase (Fig. 2A). There were relationships between CYP27B1/1 α OHase gene expression in hMSCs and serum 25OHD, some of which depended on the level of serum 25OHD. There was significantly lower expression of CYP27B1 in hMSCs from subjects with serum 25OHD less than 20 ng/ml (0.28 ± 0.23 , $n = 6$; $P = 0.021$), compared with those from subjects with serum 25OHD between 20 and 32 ng/ml (0.67 ± 0.34 , $n = 12$) (Fig. 2B). There was a significant correlation between serum 25OHD and CYP27B1 gene expression in hMSCs from subjects with serum 25OHD from 7–20 ng/ml ($r = 0.893$; $P < 0.0001$; $n = 7$). In addition, there was a trend for an inverse correlation between CYP27A1/25-hydroxylase and serum 25OHD ($P = 0.065$, Pearson correlation) (Fig. 2C). There were no associations found between serum parameters and CYP24A1/24OHase gene expression in hMSCs. Of the 24 subjects, 9% had serum PTH levels greater than 45 pg/ml, and 10% had serum 1,25(OH)₂D levels greater than 50 pg/ml. There was lower expression of CYP27B1 in hMSCs from subjects with serum PTH higher than 45 pg/ml ($P =$

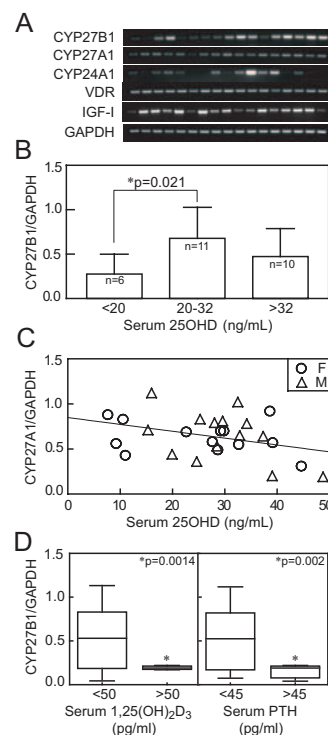


FIG. 2. Relationship between serum parameters with vitamin D metabolic enzyme gene expression in hMSCs. A, The expression of CYP27B1/1 α OHase, CYP27A1/25OHase, CYP24A1/24OHase, VDR, and IGF-I genes was assessed in hMSCs obtained from 27 consented subjects for whom clinical data were available (each lane for 17 examples shown for clarity). B, The relationship between serum 25OHD of 27 subjects and CYP27B1 gene expression in their hMSCs was assessed. Serum 25OHD less than 20 ng/ml was defined as vitamin D deficiency, and more than 32 ng/ml was defined as vitamin D sufficiency. C, There was an inverse correlation between serum 25OHD and CYP27A1/25OHase gene expression in hMSCs ($r = -0.36$; $P = 0.065$; $n = 27$; F, females; M, males). D, There was significantly lower expression of CYP27B1 in hMSCs from subjects with serum 1,25(OH)₂D higher than 50 pg/ml ($P = 0.0014$, Mann-Whitney U test) and in subjects with serum PTH higher than 45 pg/ml ($P = 0.002$).

0.002, Mann-Whitney U test) and for subjects with serum 1,25(OH)₂D higher than 50 pg/ml ($P = 0.0014$, Mann-Whitney U test) (Fig. 2D).

In vitro CYP27B1 gene expression and 1 α -hydroxylase activity in hMSCs

hMSCs from a vitamin D-deficient subject (72-yr-old female, 10.5 ng/ml serum 25OHD) were treated with or without 25OHD₃ (10 nM) with or without CYP inhibitor ketoconazole (10 μ M) for 24 h. The hMSCs expressed CYP27B1, which was up-regulated by 25OHD₃. Both basal and up-regulated levels of CYP27B1 were inhibited by ketoconazole (Fig. 3A). The 1 α -hydroxylase activity was assessed in hMSCs from the same subject by measuring 1,25(OH)₂D₃ in the medium with an ELISA. Media were collected from confluent hMSCs treated for 24 h with or without 1 μ M 25OHD₃ as exogenous substrate with or without 10 μ M ketoconazole (Fig. 3B). The 1 α -hy-

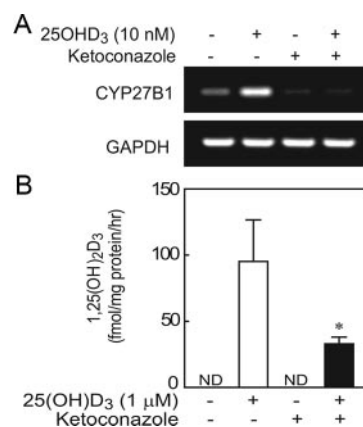


FIG. 3. Effects of CYP inhibitor ketoconazole on CYP27B1/1 α OHase gene expression and activity in hMSCs from a 72-yr-old vitamin D-deficient female subject. A, After 24 h treatment, CYP inhibitor ketoconazole (10 μ M) blocked the stimulation by 25OHD₃ (10 nM) on CYP27B1/1 α OHase gene expression. B, Ketoconazole (10 μ M) significantly inhibited synthesis of 1,25(OH)₂D₃ in the presence of 25OHD₃ (*, $P < 0.05$; $n = 3$). There was no detectable (ND) 1,25(OH)₂D₃ in cultures without 1 μ M 25OHD₃ exogenous substrate.

droxylase activity was expressed as biosynthesized 1,25(OH)₂D₃ in medium. In the presence of ketoconazole, biosynthesis of 1,25(OH)₂D₃ was 34.6% of control with only exogenous substrate ($P < 0.05$, Mann-Whitney U test).

Constitutive expression of IGF-I in hMSCs

Expression of the growth factors IGF-I, TGF- β 1, FGF-2, and PDGF- β 1 was evaluated in all hMSCs because of their potential roles in skeletal homeostasis. None of the

growth factors showed correlations with serum parameters. Of those growth factors, IGF-I was the only growth factor correlated with expression of a hydroxylase. There was a significant correlation between constitutive expression levels of IGF-I and CYP27A1 ($r = 0.71$; $P = 0.0003$). These findings prompted *in vitro* experiments for regulation of and by IGF-I.

In vitro, dose-dependent effects of 25OHD₃ and 1,25(OH)₂D₃ on CYP27B1/1 α OHase and IGF-I gene expression

hMSCs obtained from a vitamin D-deficient subject (72-yr-old female) were cultured to confluence and treated with or without 25OHD₃ or 1,25(OH)₂D₃ for 24 h. The effect of 25OHD₃ on CYP27B1 (Fig. 4A) and IGF-I (Fig. 4B) gene expression depended on the concentration of 25OHD₃. There was a dose-dependent increase in CYP27B1 and IGF-I gene expression with treatment from 0.001 nM (0.0004 ng/ml) to 10 nM (4 ng/ml) of 25OHD₃. With more than 10 nM (4 ng/ml) 25OHD₃, there was a decline in magnitude of stimulation of CYP27B1. There was a significant correlation between IGF-I and CYP27B1 gene expression in all of those samples ($P = 0.0063$; $n = 11$) (Fig. 4C). Twenty-four hours treatment with 1,25(OH)₂D₃ stimulated IGF-I and CYP24A1, down-regulated CYP27B1, and had no detectable effect on CYP27A1 gene expression (Fig. 4D). In addition, 25OHD₃ (10 nM) up-regulated by 8-fold the expression of CYP24A1 compared with vehicle control.

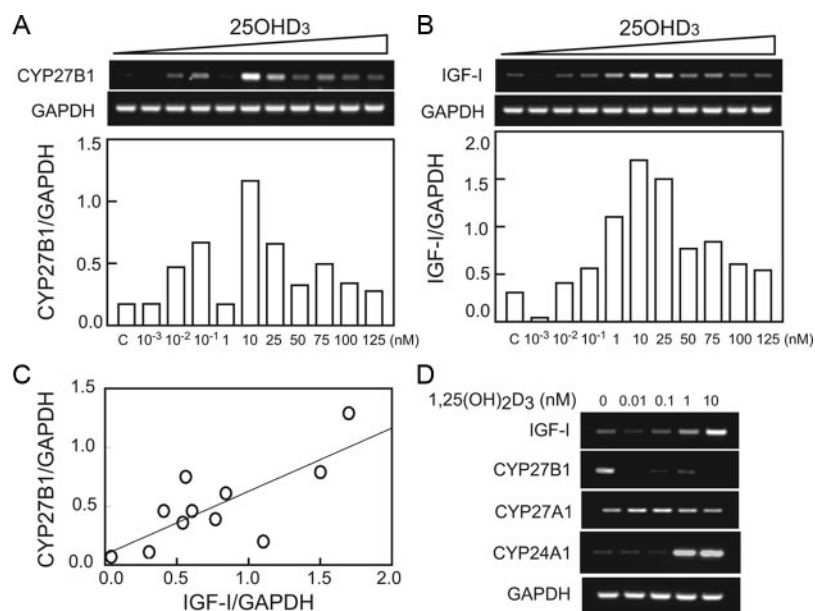


FIG. 4. *In vitro* effects of 25OHD₃ and 1,25(OH)₂D₃ on CYP27B1 and IGF-I gene expression in hMSCs obtained from a vitamin D-deficient subject (72-yr-old female). After 24 h treatment with 25OHD₃, expression levels of CYP27B1 (A) and IGF-I (B) were modulated in dose-dependent manner. C, There was a significant correlation between IGF-I and CYP27B1 gene expression in those samples ($r = 0.76$; $P = 0.0063$; $n = 11$). D, IGF-I, CYP27B1, and CYP24A1 but not CYP27A1 were modulated by 1,25(OH)₂D₃ (0.01–10 nM).

In vitro effects of IGF-I on CYP27B1/1 α OHase gene expression and activity and osteoblast differentiation in hMSCs

The effects of IGF-I on CYP27B1 gene expression in hMSCs were determined. Treatment with IGF-I for 24 h stimulated CYP27B1 expression in hMSCs in a dose-dependent manner above 5 ng/ml (Fig. 5A). MSCs from another subject were used to measure *in vitro* biosynthesis of 1,25(OH)₂D₃. Cells were incubated for 24 h with or without 1 μ M 25OHD₃ exogenous substrate with or without 100 ng/ml IGF-I in serum-free α -MEM (1% ITS⁺) with antioxidant [10 μ M 1,2-dianilinoethane (N,N'-diphenylethylene-diamine)]. IGF-I significantly increased 1,25(OH)₂D₃ biosynthesis by 40% ($P < 0.05$; $n = 4$, Mann-Whitney U test) in the presence of exogenous substrate

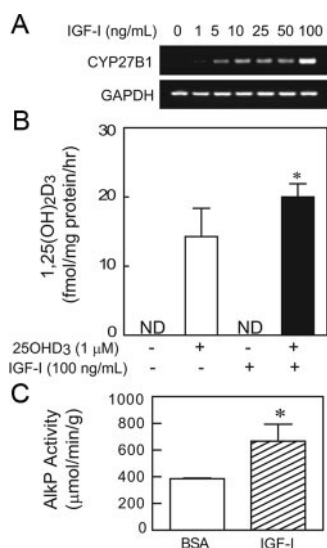


FIG. 5. Effects of IGF-I on CYP27B1/1 α OHase gene expression and 1 α -hydroxylase activity and AlkP activity in hMSCs. A, After 24 h treatment, IGF-I (1–100 ng/ml) dose-dependently stimulated CYP27B1/1 α OHase gene expression in hMSCs obtained from a 72-yr-old female subject (72F). B, IGF-I (100 ng/ml) significantly stimulated 1 α -hydroxylase activity, shown as 1,25(OH)₂D₃ biosynthesis in hMSCs obtained from a 60-yr-old male subject (*, $P < 0.05$; $n = 4$). C, Osteoblast differentiation of hMSCs was assessed with AlkP activity assays in a 12-well plate after 7 d culture with or without IGF-I (1 ng/ml) in osteogenic medium (*, $P < 0.05$; $n = 3$).

25OHD₃ (Fig. 5B). In addition, after 7 d treatment in osteogenic medium, IGF-I (1 ng/ml) stimulated AlkP activity in hMSCs by 73% ($P < 0.05$; $n = 3$, Mann-Whitney U test) (Fig. 5C).

Discussion

The classical actions of vitamin D concern mineral and skeletal homeostasis. Prolonged vitamin D deficiency has several skeletal consequences in humans. It can result in decreased bone formation and mineralization, known as rickets in children and as osteomalacia in adults. It can also lead to increased osteoclastic bone resorption that results in osteopenia or osteoporosis. Although these major effects relate to the actions of 1,25(OH)₂D₃ on intestinal calcium absorption, some information is available about direct effects on osteoblasts (33). Human trabecular bone cells responded *in vitro* to exogenous 1,25(OH)₂D₃ by increasing expression of the bone matrix genes osteocalcin and bone sialoprotein-1 (34). Whereas circulating 1,25(OH)₂D originates in the kidney, local production has been shown for normal osteoblasts (35, 36) and for human osteosarcoma cell lines (37). The differentiation of hMSCs to osteoblasts is enhanced by 1,25(OH)₂D₃ (25), but there is no information about vitamin D metabolism in hMSCs. Our finding that both 25OHD₃ and 1,25(OH)₂D₃ stimulated osteoblastogenesis, as measured by AlkP, in the

majority of preparations of hMSCs and, in some cases, to equal extents raised the possibility of 1 α -hydroxylation in human marrow stromal cells.

Vitamin D is activated in the skin or absorbed from the gastrointestinal tract and is metabolized to 25OHD in the liver (providing steady-state levels of this metabolite) and then to 1, 25(OH)₂D in the kidney by the CYP27B1 enzyme. The renal 1 α -hydroxylase is regulated by the circulating concentrations of calcium, PTH, and phosphorus. Emerging data now suggest that other cell types have the ability to generate and inactivate vitamin D, although there are no data for human marrow stromal cells. To gain new information about vitamin D metabolism in human marrow stromal cells, we used *in vivo* and *in vitro* approaches to test whether hMSCs participate in vitamin D metabolism and whether expression of vitamin D hydroxylases in hMSCs *in vitro* are influenced by the vitamin D status of the individual from whom the hMSCs were obtained and to evaluate potential mechanisms of regulation of vitamin D metabolic enzymes in hMSCs.

To test whether expression of vitamin D hydroxylases in hMSCs *in vitro* are influenced by the vitamin D status of the individual from whom the hMSCs were obtained, we analyzed the clinical data of 27 enrolled subjects with vitamin D-related gene expression in their hMSCs. As reported in the literature (38–40), our clinical data also demonstrated inverse correlations between serum 25OHD and percent body fat, BMI, and serum PTH. Although it had previously been assumed that osteoarthritis may be associated with high bone density, and in fact, many of the subjects in this study did have high T-scores and high BMIs, low T-scores and osteoporosis are now recognized to be common in patients with osteoarthritis, especially in those with low BMI or advanced age (41–44).

The expression of 25-hydroxyvitamin D-1 α -hydroxylase (CYP27B1) was lower in hMSCs from subjects with elevated serum 1,25(OH)₂D; this may signify feedback repression. The positive correlation between serum 25OHD (7–20 ng/ml) and CYP27B1 gene expression may signify feed-forward induction. Samples from subjects with the highest levels of serum 25OHD tended to have the lowest expression of CYP27A1; this suggests feedback repression.

Human primary osteoblasts and human osteoblastic cell lines possess the molecular machinery to both respond to and metabolize 25OHD₃ (37). This study shows that osteoblast differentiation in hMSCs was stimulated by both 1,25(OH)₂D₃ and 25OHD₃ in the majority of samples; this observation suggested that 25OHD directly acts on these osteoblast precursor cells or can be activated to 1,25(OH)₂D₃ *in vitro*. It will be valuable to determine whether clinical properties of individual subjects influence the effects of 25OHD₃ on *in vitro* osteoblastogenesis.

Among the CYP isoforms that have been shown to hydroxylate vitamin D, CYP27B1/25OHD-1 α -hydroxylase hydroxylates the principal circulating vitamin metabolite, 25OHD₃, into the active hormonal form, 1,25(OH)₂D₃ (45, 46). To test whether hMSCs metabolize 25OHD₃ into 1,25(OH)₂D₃, we analyzed gene expression and activity of 25OHD 1 α -hydroxylase (CYP27B1) in hMSCs. The *in vitro* data show that CYP27B1 was expressed in hMSCs and was up-regulated by exogenous 25OHD₃. Moreover, the 1,25(OH)₂D assay showed that hMSCs have the capacity to convert 25OHD into 1,25(OH)₂D. Ketoconazole, a recognized CYP inhibitor (47), inhibited both gene expression of CYP27B1 and biosynthesis of 1,25(OH)₂D in hMSCs. The up-regulation of CYP27B1 by 25OHD₃ and down-regulation by 1,25(OH)₂D₃ show a feed-forward amplification mechanism and feedback repression, respectively, at the level of gene expression. Increased substrate, 25OHD, and increased 1 α -hydroxylase, CYP27B1, would result in increased synthesis of 1,25(OH)₂D, which in turn, would down-regulate the enzyme, as suggested by the lower levels of CYP27B1 with the highest levels of added 25OHD₃. It is notable that the range of 25OHD correlated with up-regulation of CYP27B1 *in vitro* is similar to the *in vivo* range for correlation between serum 25OHD and constitutive expression of CYP27B1 in hMSCs. Further evidence of regulation of vitamin D metabolism in marrow stroma is that the higher levels of added 25OHD₃ or 1,25(OH)₂D₃ up-regulated 24-hydroxylase (CYP24A1), which initiates inactivation of the metabolites and prevents risk of hypercalcemia. Regulation of synthesis and inactivation of 1,25(OH)₂D₃ in hMSCs is similar to that described in skin and bone cells (17, 47).

Skeletal IGF-I may play multiple roles in skeletal homeostasis. First, both 25OHD₃ and 1,25(OH)₂D₃ stimulated IGF-I gene expression in hMSCs. In a number of other experimental systems, 1,25(OH)₂D₃ stimulates IGF-I in osteoblasts and preosteoblasts, and thus IGF-I may mediate osteoblastogenic actions of 1,25(OH)₂D₃ (48–50). Much of the information about vitamin D's effects on bone concern *in vitro* actions of 1,25(OH)₂D₃ to decrease proliferation and increase expression of vitamin D response genes such as osteocalcin and bone sialoprotein (36).

Second, our data show that exogenous IGF-I regulated synthesis of 1,25(OH)₂D₃ by hMSCs. IGF-I stimulated CYP27B1/1 α OHase gene expression and synthesis of 1,25(OH)₂D₃, evidence that IGF-I may be involved in vitamin D metabolism in hMSCs. Other *in vivo* and *in vitro* studies have suggested that IGF-I regulates the renal production of 1,25(OH)₂D₃ (10–14). Finally, IGF-I stimulated osteoblast differentiation of hMSCs in osteogenic

medium. Although it has been suggested that 1,25(OH)₂D effects on osteoblast (49) and chondrocyte (51) differentiation may be mediated through the IGF-I, studies have shown confounding effects of duration of treatment (52), influence of dexamethasone (53), and stage of development (50). Clearly, other components of the skeletal IGF system would also be involved (50). For example, in a pilot study of marrow samples, we reported that IGF-I, its binding proteins, and IGF-binding protein-3 protease were secreted by human marrow stromal cells and that there was an age-related increase in constitutive secretion of IGF-binding protein-3 with a notable exception for marrow from a woman receiving estrogen replacement therapy at the time of surgery (54). More detailed analysis of the relationship between clinical parameters and marrow regulation of the IGF system would advance understanding of the physiological roles of factors in the bone microenvironment. We propose that circulating 25OHD, by virtue of its local conversion to 1,25(OH)₂D catalyzed by CYP27B1 in hMSCs, amplifies vitamin D signaling through IGF-I up-regulation, which in turn induces CYP27B1 in a feed-forward mechanism to potentiate osteoblast differentiation initiated by IGF-I.

In summary, to our knowledge, this is the first evidence of enzymes involved in vitamin D metabolism being present in and regulated in hMSCs, cells shown to differentiate to osteoblasts in response to 1,25(OH)₂D (25). In the studies using marrow obtained from subjects for whom we obtained clinical data, there were correlations between circulating serum 25OHD, 1,25(OH)₂D, and PTH levels with the expression of vitamin D metabolic enzymes in their hMSCs. The *in vitro* data show regulation of the hydroxylases in marrow stromal cells by classic substrate induction and feedback suppression at the level of gene expression. Moreover, IGF-I appears to play an important amplification role in regulation of vitamin D metabolic enzymes and osteoblast differentiation in hMSCs. There is great interest in the significance of extrarenal hydroxylases and synthesis of 1,25(OH)₂D₃ (55). This study provides new information about local production of 1,25(OH)₂D₃ and expression of VDR and vitamin D hydroxylases in human bone marrow and suggests how paracrine/autocrine networks in the bone microenvironment may be regulated systemically and locally.

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