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Effects of 25-Hydroxyvitamin D3 on Proliferation and Osteoblast Differentiation of Human Marrow Stromal Cells Require CYP27B1/1α-Hydroxylase

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ABSTRACT

1,25-Dihydroxyvitamin D3 [1,25(OH)2D3] has many noncalcemic actions that rest on inhibition of proliferation and promotion of differentiation in malignant and normal cell types. 1,25(OH)2D3 stimulates osteoblast differentiation of human marrow stromal cells (hMSCs), but little is known about the effects of 25-hydroxyvitamin D3 [25(OH)D3] on these cells. Recent evidence shows that hMSCs participate in vitamin D metabolism and can activate 25(OH)D3 by CYP27B1/1α-hydroxylase. These studies test the hypothesis that antiproliferative and prodifferentiation effects of 25(OH)D3 in hMSCs depend on CYP27B1. We studied hMSCs that constitutively express high (hMSCshi-1α) or low (hMSCslo-1α) levels of CYP27B1 with equivalent expression of CYP24A1 and vitamin D receptor. In hMSCshi-1α, 25(OH)D3 reduced proliferation, downregulated proliferating cell nuclear antigen (PCNA), upregulated p21Waf1/Cip1, and decreased cyclin D1. Unlike 1,25(OH)2D3, the antiapoptotic effects of 25(OH)D3 on Bax and Bcl-2 were blocked by the P450 inhibitor ketoconazole. The antiproliferative effects of 25(OH)D3 in hMSCshi-1α and of 1,25(OH)2D3 in both samples of hMSCs were explained by cell cycle arrest, not by increased apoptosis. Stimulation of osteoblast differentiation in hMSCshi-1α by 25(OH)D3 was prevented by ketoconazole and upon transfection with CYP27B1 siRNA. These data indicate that CYP27B1 is required for the antiproliferative and prodifferentiation effects of 25(OH)D3 on hMSCs: Those effects were not seen (1) in hMSCs with low constitutive expression of CYP27B1, (2) in hMSCs treated with ketoconazole, and (3) in hMSCs in which CYP27B1 expression was silenced. Osteoblast differentiation and skeletal homeostasis may be regulated by autocrine/paracrine actions of 25(OH)D3 in hMSCs.

KEY WORDS: BONE MARROW STROMAL CELLS; VITAMIN D; PROLIFERATION; OSTEOBLAST DIFFERENTIATION; APOPTOSIS

Introduction

Vitamin D is an important regulator of mineral and bone metabolism, and it is now appreciated that its metabolites and analogues have many other actions. Calcitriol, or 1α,25-dihydroxyvitamin D3 [1,25(OH)2D3], is the most active metabolite, with high affinity for the nuclear vitamin D receptor (VDR). It is produced in the kidney by the 1α-hydroxylation of the precursor 25-hydroxyvitamin D3 [25(OH)D3] by the cytochrome P450 enzyme CYP27B1/1α-hydroxylase. Hydroxylation of vitamin D metabolites at the carbon 24 position by 25-hydroxyvitamin D–24-hydroxylase (CYP24A1) is the first step in their inactivation and excretion. Basal expression of CYP24A1 is usually low but is highly induced by 1,25(OH)2D3. Calcitriol has major effects in inhibiting proliferation and promoting differentiation of many cell types, especially tumor cells such as human breast cancer cells, colon sarcoma cells, prostate cancer cells, colorectal adenoma, and carcinoma cells. Epidemiologic and experimental studies also indicate that 1,25(OH)2D3 has antitumor effects; those effects are attributed to the inhibition of proliferation, arrest of cell cycle, increase in apoptosis, and induction of differentiation. The antiproliferative and prodifferentiation effects of 1,25(OH)2D3 also have been demonstrated for some nonmalignant cell types, such as human peripheral monocytes. Little is known, however, about the effects of 25(OH)D3 on cell proliferation and differentiation.

In addition to kidney tubule cells, other human cells, notably osteoblasts and their progenitors in the bone marrow, produce 1,25(OH)2D3. Bone cells participate in vitamin D metabolism and also are targets of 1,25(OH)2D3 action. The differentiation of human marrow stromal cells (hMSCs, a mesenchymal stem cell) and rat osteogenic ROS 17/2 cells require CYP27B1/1α-hydroxylase.
to osteoblasts is stimulated by 1,25(OH)\(_2\)D\(_3\). Less is known about
the effects of 25(OH)D\(_3\) on bone cells. In recent studies with
freshly isolated hMSCs from 19 subjects, 1,25(OH)\(_2\)D\(_3\) stimulated
osteoblast differentiation in all samples, and 25(OH)D\(_3\) did so in
two-thirds of them\(^{17}\). The variability in response to 25(OH)D\(_3\)
may be due to differences in expression of CYP27B1. The combined
presence of CYP27B1 and VDR indicates possible autocrine/paracrine roles for 25(OH)D\(_3\) in hMSCs. This study tests
the hypothesis that the antiproliferative and prodifferentiation
effects of 25(OH)D\(_3\) in hMSCs depend on CYP27B1.

Materials and Methods

Cells and reagents

Bone marrow samples were obtained with institutional review
board approval as femoral tissue discarded during primary hip
arthroplasty for osteoarthritis. A series of samples from
22 subjects (average age is 58 ± 15 years) was prepared and
screened. Low-density marrow mononuclear cells were isolated
by centrifugation on Ficoll/Histopaque 1077 (Sigma, St Louis,
MO, USA)\(^{20}\). 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. The nonadherent hematopoietic stem cells
were rinsed away 24 hours after seeding, and the adhering
hMSCs were expanded in monolayer culture with standard
growth medium, phenol red–free
\(-\text{MEM}, 10\% \text{FBS-HI, 100 U/mL of penicillin, and 100} \ \mu\text{g/mL of streptomycin (Invitrogen, Carlsbad, CA, USA). All samples were used at passages 2 through 4. Some experiments used standard osteogenic medium (ie, phenol red–free} \(-\text{MEM}, 10\% \text{FBS-HI, 100 U/mL of penicillin, and 100} \ \mu\text{g/mL of streptomycin, 10 nM dexamethasone, 5 mM}
\b-glycerophosphate, and 50 \mu\text{g/mL of ascorbate-2-phosphate})
or osteogenic medium (ie, phenol red–free} \(-\text{MEM, 1\% FBS-HI, 100} \ \text{U/mL of penicillin, 100} \ \mu\text{g/mL of streptomycin, 10 nM}
dexamethasone, 5 mM \b-glycerophosphate, and 50 \mu\text{g/mL of ascorbate-2-phosphate}). After transfection with siRNA, all media
used were without 100 U/mL of penicillin and 100 \mu\text{g/mL of}
streptomycin. Reagents such as 25(OH)D\(_3\), 1,25(OH)\(_2\)D\(_3\), and
ketoconazole were purchased from Sigma; each was prepared as
a stock solution at 10\(^{-3}\) M in absolute ethanol and stored at
–80°C. In preliminary dose-finding studies (data not shown) with
Western immunoblotting, we found no responses to 1, 10,
or 100 nM 25(OH)D\(_3\) and responses to 1000 nM 25(OH)D\(_3\); thus
most experiments used 1000 nM 25(OH)D\(_3\). In all 3-day
experiments, vitamin D metabolites were added daily to control
for inactivation by 24-hydroxylation.

Table 1. Primer Sets Used for RT-PCR

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<th>Accession number</th>
<th>Primer name</th>
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| NM_000785.3 | CYP27B1 | F = GCTACACGAGCTGAGTGCCAGGGC  
  R = AGCGGGCCAGGAGACCTGCGGAGCC | 252 |
| NM_001128915.1 | CYP24A1 | F = GCAGCCTAGTGCAGATT  
  R = ATTACCCGAAGCTGTTG | 335 |
| NM_001017535.1 | VDR | F = AGCCCTCAATAGGAG CACTCCAAAG  
  R = AGGGTTGAGGGCTGCTGAGTA | 208 |
| NM_004324.3 | Bax | F = GAGGATGATGATGCGCCAGTGAC  
  R = CGGTGGTGGGGGTTGAGAG | 279 |
| NM_000633.2 | Bcl-2 | F = CTCTCCATGTTGTGCGGATCA  
  R = CCCAGGCGAAAAGAATGCAAGTGA | 137 |
| NM_001015051.3 | Runx2 | F = GTGGTGTTCGTTGACCCTC  
  R = CCATGCTCTGAGGACCTGAAA | 318 |
| NM_000478.4 | AlkP | F = GCAGAAGAGGTAATTTGAGCAGC  
  R = TTGCAAGAGGAAATCTTTCAGC | 369 |
| NM_004967 | BSP | F = TCAGCATTCTGGGAAATGCC  
  R = GAAGGTGTTTGTTGCGAAGT | 657 |
| NM_002046.3 | GAPDH | F = TGATGGATCAAGAAGGGTTGGAAG  
  R = TCCTGGAGGACCATGTCGGC | 240 |

RNA isolation and RT-PCR

Total RNA was isolated from human MSCs with TRIZOL reagent
(Invitrogen). For reverse-transcriptase polymerase chain reaction
(RT-PCR), 2 \mu\text{g of total RNA was reverse-transcribed into cDNA with SuperScript II (Invitrogen) following the manufacturer's instructions. Concentrations of cDNA and amplification conditions were optimized for each gene product to reflect the exponential phase of amplification. One-twentieth of the cDNA was used in each 50 \mu\text{L PCR reaction (30 to 40 cycles of 94°C for 1 minute, 55 to 60°C for 1 minute, and 72°C for 2 minutes), as described previously.}^{20}\) Gene-specific primer pairs (Table 1) for
CYP27B1\(^{17}\), CYP24A1\(^{17}\), VDR\(^{17}\), Cbfa1/Runx2 (Runx2)\(^{21}\), AlkP\(^{21}\), bone sialoprotein (BSP)\(^{21}\), Bax\(^{21}\), and Bcl-2\(^{22}\) were used for amplification. PCR products were separated by agarose gel
electrophoresis and were quantified by densitometry of captured gel images with a Kodak Gel Logic 200 Imaging System and Kodak Molecular Imaging Software following the manufacturer's instructions (Kodak Molecular Imaging Systems,
Rochester, NY, USA). Data were expressed by normalizing the densitometric units to GAPDH (internal control).

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

For comparing synthesis of 1,25(OH)₂D₃, hMSCs (three replicate wells) were cultivated in 12-well plates until confluence, and then the medium was changed to serum-free α-MEM supplemented with 1% insulin-transferrin-selenium plus linoleic-bovine serum albumin (ITS)⁺⁻, 10 μM 1,2-dianilinoethane (N,N’-diphenylethylenediamine; Sigma) and treated with or without 1000 nM 25(OH)D₃ for 24 hours. This concentration of substrate 25(OH)D₃ is customary for in vitro biosynthesis studies. (17,23) 1,2-Dianilinoethane was added to the cultures as an antioxidant. Supernatants were harvested and stored at −20°C prior to analysis for 1,25(OH)₂D₃ content. The 1,25(OH)₂D₃ levels in the media were determined quantitatively with a 1,25(OH)₂D₃ EIA kit (Immunodiagnostic Systems, Ltd., Fountain Hills, AZ, USA) 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, Mannheim, Germany). Protein concentration was determined with the BCA System (Thermo Fisher Scientific, Rockford, IL, USA). The CYP27B1 activity was expressed as biosynthesized 1,25(OH)₂D₃ in medium per milligram of protein per hour (femtomoles per milligram of protein per hour).

Proliferation

Human MSCs (hMSCsh₁₋₁a and hMSCsb₁₋₁a) at passage 2 were seeded at 3000/cm² in 12-well plates. Cells were cultured in replicate (12 replicate wells) in standard growth medium (10% FBS-HI) in the absence or presence of 1, 10, or 100 nM 1,25(OH)₂D₃ or 25(OH)D₃ for 3 days. Cells were suspended with 0.5 mL of 0.05% trypsin-ethylenediamine tetraacetic acid (Invitrogen), and cell number was determined by hemacytometer.

Western immunoblot

Human MSCs were cultured in 100-mm dishes in standard growth medium (10% FBS-HI). At 50% confluence, the cells were treated with 1, 10, or 100 nM 1,25(OH)₂D₃ or 1000 nM 25(OH)D₃ for 3 days. Whole-cell lysates were prepared with lysis buffer (150 mM NaCl, 3 mM NaHCO₃, 0.1% Triton X-100, and a mixture of protease inhibitors; Roche Diagnostics, Mannheim, Germany) and were homogenized with a pestle (Kontes, Vineland, NJ, USA) and centrifuged at 16,000g (Eppendorf centrifuge; Eppendorf, Hamburg, Germany). Protein concentration was determined with the BCA System (Thermo Fisher Scientific). Western immunoblotting was performed as described previously. In brief, proteins were resolved on 4% to 12% SDS-PAGE (NuPAGE Bis-Tris gel; Invitrogen) and transferred onto polyvinylidene fluoride membranes (PVDF; Amersham Biosciences, Piscatway, NJ, USA). The membranes were blocked with 5% nonfat milk in PBS buffer containing 0.1% Tween-20 (PBST) for 2 to 3 hours at room temperature and incubated at 4°C overnight with primary antibodies proliferating cell nuclear antigen (PCNA) (1:3000; Abcam, Cambridge, UK), CYP27B1 (H-90, 1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), β-actin (1:8000, Santa Cruz Biotechnology, Inc.), and Bax, Bcl-2, p21Waf1/Cip1, and cyclin D1 (each at 1:1000; Cell Signaling Technology, Beverly, MA, USA). After removal of the unbound primary antibodies by three 5-minute washes with PBST, the membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (1:5000) for 1 hour at room temperature and washed three times for 5 minutes with PBST. The antibody-associated protein bands were revealed with the ECL-plus Western blotting system (Amersham Biosciences).

Alkaline phosphatase (AlkP) enzymatic activity assay

For AlkP enzymatic activity assay, the concentration of serum in standard osteogenic medium (10% FBS-HI) was reduced to 1% FBS-HI to minimize possible subsequent differences in proliferation that could confound interpretation of the effects of vitamin D metabolites on osteoblastogenesis. The medium was changed every 2 days. AlkP enzyme activity was measured spectrophotometrically, as described previously. Protein concentration was determined with the BCA system (Thermo Fisher Scientific, Inc.). The AlkP enzyme activity was expressed as micromoles per minute per gram of protein, and some was calculated as the ratio of treated relative to control.

RNA interference with CYP27B1 siRNA

Transient transfection of siRNA into hMSCsh₁₋₁a was performed by electroporation with the Human MSC Nucleofector Kit (Lonza/Amaax Biosystems, Walkersville, MD, USA) with either CYP27B1 siRNA, nonsilencing control siRNA (a nonhomologous, scrambled sequence equivalent; Santa Cruz Biotechnology, Inc.), or PBS according to the manufacturer’s instructions and as described previously. In brief, hMSCsh₁₋₁a were harvested by trypsinization and resuspended at 10⁶ cells in 100 μL of Nucleofector Solution (Lonza/Amaax Biosystems) with 10 or 100 pmol of CYP27B1 siRNA. Electroporation was performed in Nucleofector II device with Program U-23 (Lonza/Amaax Biosystems). Immediately after electroporation, the cells were transferred to 60-mm dishes or 12-well plates in phenol red–free α-MEM and 10% FBS-HI. Some cells were collected at 80% confluence for RT-PCR or Western immunoblot analysis to determine the expression of CYP27B1 siRNA. Some cells that were cultured until confluent in the 12-well plates were treated with or without 1000 nM 25(OH)D₃ in serum-free α-MEM supplemented with 1% ITS⁻¹, 10 μM 1,2-dianilinoethane (N,N’-diphenylethylenediamine) for 24 hours to assess 1α-hydroxylase activity. Cellular 1,25(OH)₂D₃ production was determined by EIA as described under “In vitro biosynthesis of 1,25(OH)₂D₃ by hMSCs.” At 24 hours after electroporation, some cells were treated with either 25(OH)D₃ (1000 nM) or vehicle control (ethanol) daily in standard growth medium (10% FBS-HI) for another 72 hours for RT-PCR assays. When some cells in the 12-well plates were nearly 80% confluent, the medium was changed to the osteogenic medium with 1% FBS-HI/100 μM 1,25(OH)₂D₃ for 7 days for assessment of AlkP enzymatic activity as another index of osteoblast differentiation.

Statistical analysis

Experiments were performed at least in triplicate. Group data are presented as mean ± SEM unless otherwise indicated. Quantitative data were analyzed with nonparametric tools, either the Mann-Whitney test 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 $p < .05$ was considered significant.

Results

Expression of CYP27B1 and CYP24A1 genes and 1α-hydroxylase activity in hMSCs

Gene expression analysis with hMSCs from 22 subjects showed a wide range of constitutive expression of CYP27B1 (Fig. 1A, showing 7 representative samples). Two samples of hMSCs were selected for detailed studies, having either high (hMSCshi-1α, from a 42-year-old man) or low (hMSCslo-1α, from a 46-year-old man) levels of CYP27B1, with equivalent expression of CYP24A1 and VDR (Fig. 1B). Their activity for 1α-hydroxylation was compared by measuring production of 1,25(OH)2D3. Biosynthesis of 1,25(OH)2D3 in the hMSCshi-1α was 2.98-fold greater than in the hMSCslo-1α (4433.4 versus 1487.9 fmol/mg protein per hour, $p < .0001$; Fig. 1C). Upregulation of CYP24A1 by 1,25(OH)2D3 (1, 10, and 100 nM) or 25(OH)D3 (1000 nM) treatment was equivalent in both specimens of hMSCs (Fig. 1D).

Relative antiproliferative effects of 25(OH)D3 and 1,25(OH)2D3 on hMSCs

Two samples of hMSCs were cultured for 3 days after seeding in standard growth medium (10% FBS-HI). There was less cellularity in cultures of both hMSCshi-1α and hMSCslo-1α treated with 100 nM 1,25(OH)2D3 compared with vehicle control. In contrast, only the hMSCshi-1α were inhibited by 25(OH)D3 (Fig. 2A).
was a dose-dependent inhibition of proliferation with 1,25(OH)₂D₃ for both cell preparations (Fig. 2B). Both 25(OH)D₃ and 1,25(OH)₂D₃ inhibited proliferation of hMSCs hi-1; there was a significant inhibition of proliferation of hMSCs hi-1 at 100 nM of 25(OH)D₃ (56% of control cell number, \( p < .001 \)) and 1,25(OH)₂D₃ (50%; \( p < .001 \)). In contrast, hMSCs lo-1 were resistant to 25(OH)D₃ (96%) yet were inhibited by 1,25(OH)₂D₃ (17%, \( p < .001 \); Fig. 2B). Consistent with the effects on cell numbers, PCNA was downregulated by 1,25(OH)₂D₃ in hMSCs hi-1 and in hMSCs lo-1 (Fig. 2C). With 25(OH)D₃ treatment, PCNA in hMSCs hi-1 was 64.9% of control, but for hMSCs lo-1, PCNA was equivalent to control. With hMSCs hi-1, both 25(OH)D₃ and 1,25(OH)₂D₃ downregulated cyclin D1 and upregulated the negative regulator p21\(^{WAF1/Cip1} \) (Fig. 2D). In hMSCs lo-1, the effects of 1,25(OH)₂D₃ on cell cycle regulators were similar to those for hMSCs hi-1, but there were no effects by 25(OH)D₃.

Relative effects of 25(OH)D₃ and 1,25(OH)₂D₃ on Bax/Bcl-2 ratios in hMSCs

Mechanisms involved in the relative effects of 25(OH)D₃ and 1,25(OH)₂D₃ were studied by analysis of expression of apoptosis-associated proteins. First, effects of metabolites were compared in hMSCs hi-1 and hMSCs lo-1. In hMSCs hi-1, both 25(OH)D₃ and 1,25(OH)₂D₃ induced a downregulation of Bax and an upregulation of the Bcl-2 protein (Fig. 3A); these effects resulted in lower Bax/Bcl-2 ratios with 100 nM 1,25(OH)₂D₃ (20% compared with vehicle control) and with 1000 nM 25(OH)D₃ (43%). In hMSCs lo-1, the Bax/Bcl-2 ratio was lower with 100 nM 1,25(OH)₂D₃ (19%), but there was essentially no effect by 25(OH)D₃ (95%). The effects on mRNA levels of Bax and Bcl-2 (Fig. 3B) corresponded with the changes observed for protein levels (Fig. 3A).

As a second approach, the cytochrome P450 inhibitor ketoconazole was used to determine the importance of hydroxylation on 25(OH)D₃ effects on proliferation. Ketoconazole (10 μM) diminished the effects of 25(OH)D₃ (1000 nM) and not the effects of 1,25(OH)₂D₃ (10 nM) on Bax and Bcl-2 in hMSCs hi-1 (Fig. 3C). In the presence of 25(OH)D₃, the Bax/Bcl-2 ratio was 27% of that with vehicle control, and the decrease by 25(OH)D₃ was blocked by ketoconazole (90%). In the presence of 1,25(OH)₂D₃, the Bax/Bcl-2 ratio was 36% of that with vehicle control, similar to that with 1,25(OH)₂D₃ and ketoconazole (37%).

Relative effects of 25(OH)D₃ and 1,25(OH)₂D₃ on osteoblast differentiation in hMSCs

Regulation of osteoblast differentiation was quantified first by AlkP enzymatic activity assays in osteogenic medium with 1% FBS-HI (Fig. 4A). In hMSCs hi-1, there was similar stimulation of AlkP activity by 25(OH)D₃ (2.16-fold, \( p = .0003 \)) and by 1,25(OH)₂D₃ (1.77-fold, \( p < .0001 \)). In contrast, with hMSCs lo-1, 25(OH)D₃ had no effect (0.96-fold, \( p = .577 \)), and 1,25(OH)₂D₃ stimulated AlkP activity (1.86-fold, \( p < .0001 \)).

Osteoblast differentiation was also monitored by osteoblast signature genes (ie, Runx2, AlkP, and BSP) after transfer to standard osteogenic medium (10% FBS-HI) or after addition of 1,25(OH)₂D₃ to standard growth medium (10% FBS-HI). As expected, there was time-dependent upregulation of Runx2, AlkP, and BSP in hMSCs hi-1 in standard osteogenic medium (Fig. 4B). Addition of 1,25(OH)₂D₃ to standard growth medium also upregulated osteoblast genes in hMSCs lo-1 (Fig. 4C). Addition of 25(OH)D₃ to standard growth medium also upregulated osteoblast genes in hMSCs hi-1, but its effect was diminished by ketoconazole (Fig. 4D).

Effect of CYP27B1 siRNA on the stimulation of osteoblast differentiation by 25(OH)D₃

As another approach to assess the mechanism by which 25(OH)D₃ can stimulate osteoblast differentiation, hMSCs hi-1 were engineered to have reduced constitutive expression of CYP27B1. There were no noticeable differences in cell density or appearance of control cells (electrophoresis with PBS), cells treated with nonsilencing control siRNA, and cells with 10 or 100 pmol CYP27B1 siRNA (Fig. 5A). Transient transfection of CYP27B1 siRNA into hMSCs hi-1 resulted in reductions of CYP27B1 mRNA (2% of control; Fig. 5B) and CYP27B1 protein (11% of control; Fig. 5C). No effect was shown with a nonsilencing, scrambled siRNA sequence (lane NC in Fig. 5B, C). The amount of 1,25(OH)₂D₃ synthesized by the cells transfected with CYP27B1
siRNA was 22% of that for cells transfected with nonsilencing siRNA (1075 versus 4786 fmol/mg protein per hour, p < .0001; Fig. 5D). Treatment with 25(OH)D3 upregulated Runx2, AlkP, and BSP in both control preparations of hMSCs. With cells transfected with CYP27B1 siRNA, however, 25(OH)D3 had no effect on osteoblast genes (Fig. 5E). As a functional marker of osteoblast differentiation, we measured AlkP enzymatic activity after 7 days in osteogenic medium (1% FBS-HI). Whereas 25(OH)D3 stimulated AlkP activity of control cells (1.87-fold, after 7 days in osteogenic medium (1% FBS-HI). Whereas effect on osteoblast genes (Fig. 5A) and osteoblast signature genes (Runx2, AlkP, and BSP) and GAPDH in hMSCs after 3 days in the absence or presence of 10 nM 25(OH)D3 in serum-free α-MEM supplemented with 1% ITS-1, 10 μM 1,2-dianilinoethane (N/V-diphenylethylene diamine) for 24 hours. Cellular 1,25(OH)2D3 production was determined by EIA as described under “In vitro biosynthesis of 1,25(OH)2D3 by hMSCs.” Results are shown as the mean ± SEM (3 replicate wells). There was no detectable (ND) 1,25(OH)2D3 in cultures without 1000 nM 25(OH)D3 exogenous substrate. ***p < .001. (E) Gel electrophoretogram shows Runx2, AlkP, BSP, and GAPDH in controls and in transfected hMSCs in and in transfected cells. (C) Western immunoblot shows CYP27B1 and β-actin protein levels in controls and in transfected cells. (D) Cells transfected with nonsilencing siRNA and 100 pmol of CYP27B1 siRNA were treated with or without 1000 nM 25(OH)D3 in serum-free α-MEM supplemented with 1% ITS-1, 10 μM 1,2-dianilinoethane (N/V-diphenylethylene diamine) for 24 hours.

CYP27B1 expression was found to be related to the vitamin D status(17) and, more recently, to age(25) of the subjects. There is growing evidence that hMSCs(17) and human bone cells(26) are both sources and targets of 1,25(OH)2D3, and thus vitamin D may have multiple autocrine/paracrine actions in bones.

It was important to control for 24-hydroxylation in these studies because differences in inactivation of added vitamin D metabolites could confound interpretation. The activities of CYP27B1 and CYP24A1 are important for the maintenance of appropriate levels of 1,25(OH)2D3 and 25(OH)D3. Therefore, two

Discussion

This study used three approaches to examine the role of CYP27B1 on the effects of 25(OH)D3 in hMSCs. First, we compared cells with high and low constitutive expression of CYP27B1. Finding a wide range of expression in hMSCs from 22 subjects is consistent with our previous studies.(17) The level of

Fig. 4. Comparison of effects of 25(OH)D3 and 1,25(OH)2D3 on osteoblast differentiation in hMSCs. (A) Alkaline phosphatase enzymatic activity (6 replicate wells) was measured in hMSCs in osteogenic medium (1% FBS-HI) in the absence or presence of 10 nM 25(OH)D3 (open bars) or 25(OH)D3 (closed bars) in osteogenic medium with 1% FBS-HI. Results are reported relative to control (Rx/control) with horizontal dashed line as 1.0; mean ± SEM (6 replicate wells).

Fig. 5. Effect of CYP27B1 siRNA on the stimulation of osteoblast differentiation by 25(OH)D3. Four groups were treated by electroporation with PBS (C = control), with nonsilencing control siRNA (NC), or with 10 or 100 pmol of CYP27B1 siRNA. (A) Photomicrographs show cultures of control and transfected hMSCs with or without 1000 nM 25(OH)D3 in serum-free α-MEM supplemented with 1% ITS-1, 10 μM 1,2-dianilinoethane (N/V-diphenylethylene diamine) for 24 hours. Cellular 1,25(OH)2D3 production was determined by EIA as described under “In vitro biosynthesis of 1,25(OH)2D3 by hMSCs.” Results are shown as the mean ± SEM (3 replicate wells).
hMSCs were selected and studied in detail on the basis of having extremes in expression of CYP27B1 and equivalent expression of CYP24A1 and VDR. Further, the expression of CYP24A1 was found to be regulated with equivalence in both specimens of hMSCs; this observation reduces concerns of confounding effects of 24-hydroxylation in these studies. In addition, fresh metabolites were added daily.

There were substantial differences in the synthesis of 1,25(OH)₂D₃ by hMSCs and hMSCs. The difference also held for hMSCs with and without CYP27B1 gene silencing. Although these experiments cannot be used to estimate what would be the steady-state concentration of 1,25(OH)₂D₃ in the bone marrow in different subjects whose cells have high or low expression of CYP27B1, they provide evidence for a potential autocrine/paracrine role for 25(OH)D₃ metabolism in osteoblast differentiation. Similar ideas have been proposed for 25(OH)D₃ metabolism in regulating bone matrix differentiation by differentiated human osteoblasts.23

There was dose-dependent inhibition of proliferation by 25(OH)D₃ with hMSCs that had a high level of expression of CYP27B1; 25(OH)D₃ reduced their proliferation and down-regulated PCNA. There is some information about antiproliferative actions of 25(OH)D₃ in other human cell types. In human primary prostate epithelial cells that expressed CYP27B1, low concentrations of 25(OH)D₃ suppressed cell growth.2⁵ In prostatic cancer cells lacking CYP27B1, 25(OH)D₃ failed to mediate antiproliferative action.2⁸ There are several mechanisms mediating the antiproliferative effects of 1,25(OH)₂D₃. In U937 myelomonocytic cells, 1,25(OH)₂D₃ induces an arrest in the G₁ phase of the cell cycle that depends on up-regulation of the cyclin-dependent kinase inhibitor p21Waf1/Cip1.2⁹ More recently, p21Waf1/Cip1 was shown to be a primary antiproliferative mediator for the VDR in the presence of its ligand, 1,25(OH)₂D₃.3⁰ Cyclin D1 is increased in dividing cells during the G₁ phase and is necessary for the transition from G₁ to S phase.3¹ Vitamin D decreases cyclin D1 abundance and/or activity by different mechanisms in different cell types. For example, in human epidermoid A431 cells, 1,25(OH)₂D₃ inhibited transforming growth factor α (TGF-α)/endothelial growth factor receptor (EGFR) transactivation of cyclin D1.3² We found that 25(OH)D₃ downregulated cyclin D1 and upregulated the negative regulator p21Waf1/Cip1 in hMSCs.3³ In contrast, 25(OH)D₃ had no such effects in hMSCs. The upregulation of p21Waf1/Cip1 and decreased expression of cyclin D1 in hMSCs provide mechanisms for the antiproliferative effect of 25(OH)D₃.

1,25(OH)₂D₃ also affects the levels of proapoptotic (ie, Bax and Bak) and antiapoptotic (ie, Bcl-2 and Bcl-XL) proteins, resulting in apoptosis in several tumor models, including human carcinomas of the breast, colon, and prostate.3⁴ To investigate the effects of 1,25(OH)₂D₃ on the expression of Bax and Bcl-2, we used a pan-cytochrome P450 inhibitor ketoconazole, not like the effects of 1,25(OH)₂D₃, which were not affected by ketoconazole. The antiapoptotic effects of 1,25(OH)₂D₃ or 25(OH)D₃ in hMSCs are different from the proapoptotic effects in some cancer cells and are similar to the effects in other cell types. In ovarian cancer cells, 1,25(OH)₂D₃ inhibits apoptosis that is mediated by death receptors.3⁵ In rat osteoblast-like osteosarcoma UMR 106 cells, 1,25(OH)₂D₃ elicited antipapoptotic effects by decreasing the Bax/Bcl-2 ratio.1¹¹ There are other antipapoptotic signals, as was reported for nongenotropic mechanisms in osteoblasts and osteocytes.3⁶ In sum, the data indicate that the antiproliferative effects of 25(OH)D₃ in hMSCs and of 1,25(OH)₂D₃ in both samples of hMSCs are explained by cell cycle arrest and not by increased apoptosis.

Calcitriol induces differentiation of many types of benign and malignant cells.3²,3³,3⁴,3⁵,3⁶,3⁷,3⁸,3⁹,4⁰,4¹,4² Vitamin D or its analogues promote osteoblastic differentiation, as shown for osteosarcoma cell lines MG-63,4³,4⁴ HOS,4⁵,4⁶ SAOS,4⁷,4⁸ and TE8.4⁹ Osteoblast differentiation of human MSCs is stimulated by 1,25(OH)₂D₃ or 25(OH)D₃ in hMSCs. As shown here and elsewhere,1⁷,1⁸,3⁷,3⁸,4² expected, 25(OH)D₃ failed to stimulate osteoblast differentiation in hMSCs. Uroporphyrin of osteoblast genes by 25(OH)D₃ in hMSCs was diminished by ketoconazole. Thus experiments with ketoconazole indicate that both the antiproliferative and prodifferentiation effects of 25(OH)D₃ depend on CYP27B1.

Ketoconazole is a strong but differential inhibitor of both CYP24A1 and CYP27B1 and may be cytotoxic for some cells.3² Those confounders may complicate interpretation of results obtained with this agent. We therefore used the highly specific technique of RNA interference to inhibit CYP27B1 expression in hMSCs.3² The level of synthesized 1,25(OH)₂D₃ in the cells transfected with CYP27B1 siRNA was reduced to 22% of that for cells transfected with nonsilencing siRNA. Osteoblast differentiation of hMSCs was prevented upon transfection with CYP27B1 siRNA, as indicated by osteoblast signature gene expression and by AlkP enzymatic activity. These findings are consistent with those from a study with HOS human osteosarcoma cells in which silencing of CYP27B1 resulted in a suppression of 25(OH)D₃’s effects on those cells.3²,4¹

In conclusion, 25(OH)D₃ has multiple effects in normal hMSCs; it inhibits proliferation and promotes osteoblast differentiation by mechanisms similar to those for 1,25(OH)₂D₃. Our data indicate that antiproliferative and prodifferentiation effects of 25(OH)D₃ in hMSCs require 1α-hydroxylase. There are suggestions that other effects of 25(OH)D₃ in other cell types, such as induction of 24-hydroxylation in prostatic cells, may not require 1α-hydroxylase.4⁵ Three lines of evidence indicate that CYP27B1 is required for the effects of 25(OH)D₃ on hMSCs. Those effects were not seen (1) in hMSCs with low constitutive expression of CYP27B1, (2) in hMSCs treated with ketoconazole, or (3) in hMSCs in which CYP27B1 expression was silenced. These findings suggest that local osteoblast differentiation in vivo may be promoted by 25(OH)D₃ if the progenitor/precursor cells in marrow express CYP27B1/1α-hydroxylase. We found that many of hMSCs’ in vitro behaviors and baseline characteristics depend on functional features of the subjects from whom the cells were
isolated. The level of CYP27B1 expression in those cells depends on vitamin D status and can be regulated by a number of factors, including vitamin D.(17) The combined presence of CYP27B1 and the VDR in hMSCs indicates possible autocrine/paracrine roles for 25(OH)D3 to regulate osteoblast differentiation and skeletal homeostasis.

Disclosures

All the authors state that they have no conflicts of interest.

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