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Vitamin D metabolism and regulation in pediatric MSCs

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ABSTRACT

Vitamin D is crucial for mineral homeostasis and contributes to bone metabolism by inducing osteoblast differentiation of marrow stromal cells (MSCs). We recently reported that MSCs from adults demonstrate 1α-hydroxylase activity *in vitro* and express vitamin D-related genes; this raises a possible autocrine/paracrine role for D activation in pre-osteoblasts. In this studies, we tested the hypotheses that pediatric MSCs have 1α-hydroxylase activity and express vitamin D-related genes. With IRB approval, we isolated MSCs from discarded excess iliac marrow graft from 6 male and 6 female subjects (age 8–12 years) undergoing alveolar cleft repair. 1α-hydroxylation of substrate 25(OH)D_3 was measured by ELISA for 1α,25(OH)$_2$D. RT-PCR was used for gene expression. Pediatric MSCs showed a range of 1α-hydroxylase activity *in vitro*. There was constitutive expression of vitamin D receptor (VDR), megalin, D-hydroxylases (CYP27B1, CYP27A1, CYP2R1, and CYP24A1), and estrogen receptor (ER). There was 2.6-fold greater expression of CYP27B1 and 3.5-fold greater expression of CYP24A1 in MSCs from boys compared with girls. There was 2.4-fold greater expression of ERα and 3.2-fold greater expression of megalin in MSCs from boys. In preliminary studies, treatment of female pediatric MSCs with 10 nM 17β-estradiol resulted in upregulation of CYP27B1 and CYP24A1, as well as VDR, megalin, ERα, and ERβ. Treatment with 25(OH)D_3 upregulated CYP27B1, VDR, and ERα. Expression and regulation of vitamin D related genes in pediatric hMSCs reinforces an autocrine/paracrine role for vitamin D in hMSCs. Finding striking gender differences in MSCs from children was not seen with MSCs from adults and adds insight to the metabolic environment of bone and presents a research approach for investigating and optimizing pediatric bone health.

1. Introduction

The major circulating form of vitamin D is 25-hydroxyvitamin D [25(OH)D] and is activated in the kidney to 1α,25-dihydroxyvitamin D [1α,25(OH)$_2$D] by action of 1α-hydroxylase (CYP27B1) [1]. Renal CYP27B1 is tightly regulated by calcitropic hormones—parathyroid hormone (PTH) and calcitonin, which simulate it, and fibroblast growth factor 23 (FGF23) and 1α,25(OH)$_2$D, both of which inhibit it [2,3]. The discoveries of extra-renal CYP27B1 in various vitamin D target tissues raised interest in autocrine/paracrine roles of vitamin D metabolism in regulating cell function in those tissues, such as in keratinocytes, peripheral monocytes, and parathyroid cells [4].

Bone is one of the important targets of 1α,25(OH)$_2$D action, involved in bone matrix biosynthesis and calcium homeostasis. Bone cells, including human osteoblasts, osteoclasts, and osteocytes are capable of local biosynthesis of 1α,25(OH)$_2$D and express CYP27B1 [3,5]. Human adult marrow stromal cells (hMSCs) were shown to be precursors of several different cell lineages, including osteoblast, chondrocyte, adipocyte, and fibroblast [6]. Osteoblast differentiation of human MSCs is enhanced by 1α,25(OH)$_2$D [7]. We recently reported that adult hMSCs possess the vitamin D metabolic machinery, including CYP27B1, which is necessary to mediate 25(OH)D's ability to promote osteoblast differentiation [8,9]. This finding raised the possibility of an autocrine/paracrine role for vitamin D metabolism in osteoblast differentiation. Studies with MSCs from subjects between 41 and 87 years of age showed

Abbreviations: 1α,25(OH)$_2$D, 1α,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; BMI, body mass index; CYP, cytochrome P450; DBP, vitamin D binding protein; ER, estrogen receptor; FGF23, fibroblast growth factor 23; hMSC, human marrow stromal cell, a.k.a. mesenchymal stem cell; PTH, parathyroid hormone; VDR, vitamin D receptor.

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an age-related decline in constitutive expression of CYP27B1 and, accordingly, in responsiveness to 25(OH)D [10].

Those observations led to this study to test whether hMSCs from children have 1α-hydroxylase enzymatic activity and express the vitamin D metabolic machinery and related genes.

2. Materials and methods

2.1. Subjects

With IRB approval, bone marrow samples were obtained as fresh discarded excess iliac crest marrow used for the repair of alveolar cleft in patients with non-syndromic cleft lip/palate. As standard of care, autogenous grafting for an alveolar cleft is done when radiographs show the permanent maxillary canine tooth on the affected side has one-half to two-thirds root formation. Iliac crest particulate marrow was obtained from the posterior iliac crest after removal of a cortical window. Excess marrow was obtained from 6 girls and 6 boys, between 8 and 12 years of age. Criteria for exclusion included pre-existing conditions or medications that may influence bone remodeling, matrix mineralization, and hormonal regulation; no individuals were excluded in this study. Subject information such as gender, age, height, and weight was available before de-identification.

2.2. Preparation of human marrow stromal cells

hMSCs were isolated from fresh excess iliac crest marrow by centrifugation with Ficoll-Histopaque 1077 (Sigma Chemical Co., St. Louis, MO) [8,11]. This process isolates the low-density undifferentiated mononuclear cells, including stromal cells capable of adherence. Low-density cells were cultured with phenol red-free α-MEM with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37° in a humidified atmosphere of 5% CO_2 in air. The non-adherent cells were washed away at 24 h and the adherent hMSCs were expanded in monolayer culture with biweekly media changes. All samples were used at passage 2 to avoid potential differences in gene expression seen with prolonged culture and increasing cell passage [12]. Constitutive gene expression was evaluated by RT-PCR.

2.3. In vitro *biosynthesis of 1α,25(OH)$_2$D$_3$ by hMSCs*

Available hMSCs from 3 subjects (two 8-year-old girls and one 10-year-old boy) were used to assess biosynthesis of 1α,25(OH)$_2$D$_3$ at the same time. The hMSCs were cultured in 12-well plates for evaluation of constitutive 1α-hydroxylase enzymatic activity [8–10]. Upon confluence, cells were incubated for 24 h with substrate 25(OH)D$_3$ (1 μM) (Sigma) or with vehicle in serum free α-MEM supplemented with 1% ITS (Sigma) and 1,2 dianilinoethane (*N,N*-diphenylethylene-diamine) (10 μM) as an anti-oxidant [13]. Media were collected and 1α,25(OH)$_2$D was measured by ELISA (Immunodiagnostic Systems Ltd., Scottsdale, AZ, USA) according to the manufacturer's directions. Cell monolayers were lysed with buffer containing 150 mM NaCl, 3 mM $NaHCO_3$, 0.1% Triton X-100 and a mixture of protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA). Protein concentration of the lysate was measured using BCA system (Thermo Fisher Scientific Inc., Rockford, IL, USA). The 1α-hydroxylase activity was expressed as the concentration of biosynthesized 1α,25(OH)$_2$D per mg of protein per hour [8–10].

2.4. In vitro *treatments*

hMSCs from a 9-year-old girl were plated in 100-mm plates and grown to confluence in basal medium. At confluence the medium was changed to basal medium with 1% serum and 10 nM 17β-estradiol [14] for 0, 1, 2 or 3 days, with time 0 acting as the control. At intervals, cells were collected for gene expression analysis for CYP27B1, CYP27A1, CYP24A1, CYP2R1, VDR, megalin/LRP2, estrogen receptor α (ERα), and ERβ. Other dishes from the same subject were exposed to 10 nM 25(OH)D$_3$ or ethanol vehicle for 24 h in basal medium with 1% serum, for gene expression analysis.

2.5. RNA isolation, RT-PCR, and semi-quantitative analysis

Total RNA was isolated from hMSCs with TRIzol reagent (Invitrogen) according to the manufacturer's directions. For each sample, aliquots of 2 μg of total RNA were transcribed into cDNA with M-MLV reverse transcriptase (Promega). One twentieth of the cDNA was used in each 50 μl PCR reaction with Promega GoTaq Flexi DNA Polymerase. Concentration of cDNA and amplification conditions was optimized to reflect the exponential phase of amplification. Gene specific primers were used for human CYP27B1 [15], CYP27A1 [8], CYP24A1 [16], CYP2R1 [17], VDR [18], megalin/LRP2 [19]. Primers for ER-α (forward 5′-AAGGAGACTCGCTACTGTGCAG-3′ and reverse: 5′-ATCAGGATCTCTAGCCAGGCAC-3′, 625 bp), and for ER-β (forward 5′-TTCCCAGCAATGTCACTAACT-3′ and reverse 5′-TCTCTGTCTCCGCACAAGG-3′, 527 bp) were designed with Primer3 software. PCR products were resolved by 2% agarose gel electrophoresis and were quantified by densitometry of captured gel images with KODAK Gel Logic 200 Imaging System and KODAK Molecular Imaging Software (Carestream Health, Rochester, NY). Gene expression levels were normalized to GAPDH as internal control, as described [8–11]. Reverse transcription and/or amplifications were repeated to assess reproducibility of effects. For example, repeats of constitutive CYP27B1 expression showed a maximum of 9% relative difference between amplifications.

2.6. Statistical analyses

Group data are presented as mean values ± standard error of the mean. Data were analyzed by GraphPad InStat® (GraphPad Software, Inc., San Diego, CA, USA). The Kolmogorov–Smirnov test showed normality of data for constitutive gene expression levels; unpaired *t*-tests were used to compare groups. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Characteristics of the study subjects

Marrow was obtained from 6 boys and 6 girls ages 8–12 years with a mean age of 9.5 years. Boys (10.3 years, $n = 6$) were slightly older than girls (8.7 years, $n = 6$, $p = 0.01$); there was no significant correlation, however, between age and any of the outcomes measured. The average BMI was 18.3 kg/m^2 with no significant difference between boys (17.8 kg/m^2, $n = 6$) and girls (18.8 kg/m^2, $n = 6$, $p = 0.51$).

3.2. In vitro *1α-hydroxylase activity by hMSCs*

Three pediatric MSC preparations were available for simultaneous measurement of *in vitro* 1α-hydroxylase activity. They showed a range of 1α-hydroxylase enzyme activity, 68.7 to 160.3 fmol/mg protein/h (Fig. 1).

3.3. Constitutive expression of the vitamin D receptor, megalin, vitamin D-hydroxylases, and estrogen receptor in hMSCs

The constitutive levels of CYP27B1 in the specimens that had been used for *in vitro* biosynthesis of 1α,25(OH)$_2$D$_3$ corresponded