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Estradiol Formation by Human Osteoblasts via Multiple Pathways: Relation With Osteoblast Function

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Abstract The importance of estrogens in bone metabolism is illustrated by the accelerated bone loss and increase in osteoporotic fractures associated with postmenopausal estrogen deficiency. In this study, the expression and activity of the enzymes involved in estrogen metabolism in human osteoblastic cells were investigated in relation to differentiation of these cells. PCR reactions using mRNA from an in vitro differentiating human cell line (SV-HFO) were performed to assess mRNA expression of the enzymes aromatase, different subtypes of 17β-hydroxysteroid dehydrogenase (17β-HSD), and steroid sulfatase. Aromatase, sulfatase, and 17β-HSD type 2 and 4 were found to be expressed throughout differentiation. Expression of 17β-HSD type 3, however, was relatively weak, except for early time points in differentiation. Type 1 17β-HSD expression was not detected. Aromatase activity decreased during differentiation, as was demonstrated by the conversion of androstenedione (A) and testosterone (T) into estrone (E_1) and estradiol (E_2), respectively. The 17β-HSD isozymes catalysing a reductive reaction convert androstenedione and estrone into testosterone and estradiol, respectively. Their activity declined with differentiation. Analysis of 17β-HSD activity indicated both oxidative (E_2 to E_1; T to A) and reductive (E_1 to E_2; A to T) metabolism at all stages of osteoblast differentiation. Both activities declined as cells moved toward a differentiating mineralizing phenotype. However, the oxidative reaction was increasingly in favor of the reductive reaction at all times during differentiation. Sulfatase activity, as demonstrated by the conversion of estrone-sulfate into estrone, was constant during differentiation. In conclusion, we have demonstrated that all enzymes necessary for estrogen metabolism are expressed and biologically active in differentiating human osteoblasts. The activity of aromatase and 17β-HSD was found to be dependent on the stage of cell differentiation. In addition, human osteoblasts effectively convert estradiol into estrone. The efficacy of osteoblasts to synthesize estradiol may determine the ultimate change in rate of bone turnover after menopause, as well as the development of osteoporosis. Moreover, the enzymes involved in the metabolism of estradiol may form a target for intervention. J. Cell. Biochem. 75:528–537, 1999.

Key words: aromatase; 17β-hydroxysteroid dehydrogenase; sulfatase; estrone; androstenedione; testosterone; differentiation

Estrogens play an important role in the control of bone turnover and maintenance of bone mass. Estrogen deficiency after menopause is associated with increased bone turnover, resulting in a reduced bone mineral density (BMD) and an increased fracture risk. Postmenopausal hormone replacement therapy will bring bone turnover to premenopausal levels and stop further bone loss. The precise mechanisms by which estrogens regulate bone metabolism are not fully understood. Both direct effects on bone turnover and indirect effects via local factors, such as interleukins-1 and -6 (II-1, II-6) [Jilka et al., 1992; Kawaguchi et al., 1995], prostaglandins [Horowitz, 1993], and tumor necrosis factor-α (TNF-α) [Kawaguchi et al., 1995], have been reported. There appears to be no clear correlation between serum estradiol levels after menopause and the rate of bone loss [Nawata et al., 1995]. However, it has recently been

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reported that women older than 65 years of age with serum estradiol levels of 5–9 pg/ml have about a 60% lower risk of hip and vertebral fractures compared with women with undetectable estradiol levels (<5 pg/ml) [Cummings et al., 1998]. At this age, estradiol is derived from formation in peripheral tissues. These data demonstrate the importance of target tissue synthesis of estradiol in the postmenopausal state and that the capacity of bone to synthesize estradiol may be a determining factor in eventual bone turnover.

The principal enzymes involved in estrogen biosynthesis and metabolism are the aromatase cytochrome P-450 and isozymes of 17β-hydroxysteroid dehydrogenase (17β-HSD). Aromatase catalyzes the conversion of androstenedione (A) and testosterone (T) into estrone (E_1) and 17β-estradiol (E_2), respectively. The significance of aromatase and thereby estradiol synthesis for bone has been perfectly illustrated by the description of aromatase deficiency in humans [Morishima et al., 1995; Bilezikian et al., 1998]. The enzyme 17β-HSD is responsible for the interconversion of A and T and of E_1 and E_2. At least six isotypes of this enzyme have been detected that catalyze either the reductive (e.g., A to T and E_1 to E_2) or the oxidative (e.g., T to A and E_2 to E_1) reaction. The enzyme steroid sulfatase provides another pathway for local estrogen production. This enzyme converts estrone-sulfate (E_1-S) to E_1, which in turn can be converted to the biologically active E_2 by the action of 17β-HSD. The substrates for this enzyme, dehydroepiandrosterone sulfate (DHEA-S) (produced by the adrenals) and E_1-S (formed by sulfoconjugation of E_1), are the major circulation steroids [Coughtrie et al., 1998].

Aromatase activity has been demonstrated in various tissues; tissue-specific promoters are known to be involved in regulating its expression [Harada et al., 1993]. Recent studies have indicated that the skeleton may be a site of local estrogen production. The expression and activity of aromatase, 17β-HSD, and sulfatase have been demonstrated in primary cultured human bone [Nawata et al., 1995; Schweikert et al., 1995; Tanaka et al., 1996; Lea et al., 1997; Sasano et al., 1997] and several human osteosarcoma cell lines [Fujikawa et al., 1997; Purohit et al., 1992].

The main objective of this study was to identify the presence of the various enzymes involved in estradiol synthesis in an in vitro differentiating human osteoblast cell line. Osteoblasts proceed through a well-defined process of proliferation, matrix maturation, and mineralization of the extracellular matrix. It is known that gene expression and cell function change during osteoblast differentiation [Stein and Lian, 1993]. We further examined whether the activity of aromatase, 17β-HSD, and sulfatase is related to differentiation of the human osteoblast and functional changes associated with differentiation.

METHODS

Cell Culture

A human fetal osteoblast cell line, immortalized with SV-40 virus, was used (SV-HFO, generously provided by Professor M. Mori) [Arts et al., 1997]. Cells were cultured in phenol-red free α-Minimal Essential Medium (α-MEM) containing 20 mM HEPES (Sigma Chemical Co., St. Louis, MI), 10% (v/v) heat-inactivated fetal calf serum (FCS), 100 μg/ml streptomycin, and 100 IU/ml penicillin (Gibco, Paisley, UK), at 37°C in 5% CO_2. Medium was replaced every 2–3 days. Experimental cultures were grown in collagen-coated 24-well plates (0.3 μg/cm^2 collagen S type I, Boehringer Mannheim, Mannheim, Germany) seeded at a density of 1×10^4 cells per cm^2. From day 3 onward, the medium was supplemented with 10 mM β-glycerophosphate (Sigma) and 1 μM dexamethasone (9α-fluoro-16α-methylprednisolone; Sigma). The cells were cultured for 21 days. Incubations were performed after 6, 10, 14, and 21 days of culture. At 24 h before incubation, cells were washed with serum-free medium and then provided with α-MEM medium containing 10% charcoal-treated FCS. Incubations with androstenedione, testosterone, estrone, estrone-sulfate, and estradiol were carried out in α-MEM medium without serum and phenol red and supplemented with 0.1% human serum albumin (HSA). Alkaline phosphatase activity and total DNA were measured as described previously [Pols et al., 1986]. Collagen type I mRNA expression was assessed by Northern blot analysis; calcium deposition into the matrix was determined after extraction with 0.5 N HCl for 3 h, using the Sigma calcium assay.

RT-PCR for Aromatase and Sulfatase mRNA

Total RNA was isolated according to the method of Chomczynski and Sacchi [1987] and quantified spectrophotometrically at a wavelength of 260 nm. For first-strand complementary DNA (cDNA) synthesis, a 14-µl reaction mixture consisting of 0.5 µg total RNA, 50 pmol of reverse aromatase primer, 50 pmol of reverse sulfatase primer, and 100 pmol of reverse 28S primer was heated at 72°C for 8 min. After rapidly cooling on ice, it was added to 11 µl of a mixture containing 25 nmol of each dNTP (Sphaero Q, Leiden, The Netherlands), 1× reverse transcriptase buffer (Gibco), 10 mM DTT (Gibco), 5×10^{-2} U RNAse inhibitor (RNAGuard, Pharmacia Biotech, Woerden, The Netherlands), and 25 U of Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco). This reaction mixture was incubated for 60 min at 37°C, heated to 94°C for 10 min, and stored at −20°C. In order to check whether the amount of PCR product increased linearly as a function of the amount of total RNA used, a concentration curve of template RNA was made, supplemented with the appropriate amount of tRNA (Boehringer Mannheim) to maintain a total amount of 0.5 µg of RNA.

Polymerase chain reaction (PCR) amplification was performed in a 25-µl reaction mixture containing 50 ng of cDNA, 20 pmol of forward and nested reverse aromatase or sulfatase primers, 5 nmol of each dNTP, 0.5 U of *Taq* polymerase (Sphaero Q), and a buffer consisting of 75 mM $(NH_4)_2SO_4$, 300 mM Tris-HCl (pH 9.0), and 2.5 mM $MgCl_2$ for the aromatase PCR. The buffer for the sulfatase PCR consisted of 75 mM $(NH_4)_2SO_4$, 300 mM Tris-HCl (pH 10), and 2.0 mM $MgCl_2$. Aromatase PCR was started with a denaturation step at 94°C for 4 min, followed by 28 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and polymerization at 72°C for 2 min. For sulfatase PCR, an initial incubation at 94°C for 4 min was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and polymerization at 72°C for 2 min. PCR products were electrophoresed on 2.0% agarose gels containing 0.2 µg/ml ethidium bromide and photographed under ultraviolet (UV), light using a Polaroid ISO 3000/36°C film. Relative intensity of the bands was determined by a densitometric scanner, using 28S RNA to quantitate the level of aromatase and sulfatase mRNA expression.

The following primers were used:

Aromatase reverse 5′-GCAAAGTCCATACATTCTTCCAG-3′
Aromatase forward 5′-CGGCCTTGTTCGTATGGTCA-3′
Sulfatase reverse 5′-CTTTATAGATCCCATTACTTCCGCC-3′ (for cDNA synthesis)
Sulfatase forward 5′-GAACACTGAGACTCCGTTCCT-3′
Sulfatase reverse 5′CCTTTGGAAGACACTTCTTCTAC-3′
28S forward 5′-GTGCAGATCTTGGTGGTAGTAGC-3′
28S reverse 5′-AGAGCCAATCCTTATCCCGAAGTT-3′

RT-PCR for 17β-HSD Subtypes

RT of RNA was performed using a Promega Reverse Transcription System, following an adapted method. Briefly, 1 µg of total RNA and 0.5 µg of random hexamers in a final volume of 11 µl were incubated at 70°C for 5 min, then allowed to cool slowly to 25°C. Primer extension was performed at 37°C for 60 min after the addition of 1× (final concentration) reaction buffer containing 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, and 0.5 mM spermidine, 1 mM (final concentration) of each dNTP, 40 U of rRNAsin ribonuclease inhibitor, and 15 U of AMV reverse transcriptase in a final volume of 20 µl. The RT mixture was then heated to 99°C for 5 min, then to 4°C for 5 min.

PCR reactions of 20 µl were set up adding an aliquot of either 2 µl (18S) or 3 µl (17β-HSD) cDNA to an 1× (final concentration) PCR buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 µM (final concentration) of each dNTP, 0.5 µM final concentration of primers, and 1 U of *Taq* DNA polymerase (Promega, Madison, WI). All 17β-HSD PCR reactions were initiated by a denaturation step of 95°C. For 17β-HSD type 1–3 initial denaturation was followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1.5 min (type 1), at 50°C for 1.5 min (type 2) or at 58°C for 1.5 min (type 3), and polymerization (all isotypes) at 72°C for 2 min. 17β-HSD type 4 amplification was performed by 30 cycles of 95°C (1 min), 55°C (1 min) and 72°C (2 min). A final elongation step of 72°C for 7 min was included for all types 17β-HSD. RT-PCR analyses for 17β-HSD isoforms were

normalized using primers to amplify 18S. PCR reactions were set up as described above, except 2 mM $MgCl_2$ and 1 µl of 18S primers and 1 µl of 18S competimers (Quantum RNA kit; Ambion, Texas) were used. Amplification of samples was performed using an initial denaturation step of 95°C, followed by 25 cycles of 94°C (1 min), 50°C (1 min), 72°C (1 min). A final elongation step of 72°C for 2 min was also included.

The following primers for 17β-HSD were used:

17β-HSD1 forward 5′-AGG CTT ATG CGA GAG TCT GG-3′
17β-HSD1 reverse 3′-CAT GGC GGT GAC GTA GTT GG-5′
17β-HSD2 forward 5′-CTT AGC ACA GCG GAA TTT CC-3′
17β-HSD2 reverse 3′-CCA GTT TCC CAG TTT CCC TT-5′
17β-HSD3 forward 5′-ACA ATG TCG GAA TGC TTC-3′
17β-HSD3 reverse 3′-AGG TTG AAG TGC TGG TCT GC-5′
17β-HSD4 forward 5′-CTA TTG GCC AGA AAC TCC CT-3′
17β-HSD4 reverse 3′-GGA CCT TGG TTT GAA AAT GA-5′

Analysis of Aromatase, 17β-HSD, and Sulfatase Activity

Cells were cultured as described above and incubated with either A, T, E_1, E_2, or E_1-S for 4, 8, and 24 h. One-third of the total substrate concentration of 40 nM was labeled with tritum (A: 93 Ci/mmol; T: 98 Ci/mmol; E_1: 114 Ci/mmol; E_2: 84 Ci/mmol; E_1-S: 53 Ci/mmol). After incubation, the metabolites formed were identified as described previously [Eyre et al., 1998]. Briefly, following incubation, medium was removed and steroids were extracted in 2.5-ml chloroform. After evaporation of the organic phase, the steroids were redissolved in 50 µl chloroform and separated on thin-layer chromatography (TLC) plates in chloroform/ethyl-acetate (4:1 v/v). Because E_1-S is water soluble and cannot be extracted in chloroform alone, the incubation medium was saturated with NaCl; estrone and estrone sulfate were removed by two extractions in 6 vol of ethylacetate. After evaporation of the organic phase, steroids were redissolved in 50 µl of ethyl acetate and separated in two steps by thin-layer chromatography (TLC). In the first step, samples were separated in chloroform/ethyl-acetate (4:1 v:v). A further separation was then performed in ethyl-acetate/methanol/NaOH (1 M) (37.5:12.5:1 v:v). In the second TLC separation the solvent was only allowed to run halfway up the TLC plate. Conversion of tritiated steroid was measured using a Bioscan System 200 imaging TLC plate scanner (Bioscan, Edmonds, WA), which permitted quantification of the fractional conversion of the different substrates to each of the potential products. The DNA content of cells was measured, and enzymatic activity was expressed as pmol/h/mg DNA.

Analysis of Sulfatase Activity in Cell Extracts

Cells were cultured as described above. After the appropriate culture time medium was removed and the cells were washed twice with ice-cold PBS. Cells were scraped in ice-cold PBS and pelleted by centrifugation. After removal of the supernatant, the cell pellet was rapidly frozen in liquid nitrogen and stored at −80°C until later use. In order to assess sulfatase activity, 100 µl of 0.1 M Tris-maleate buffer was added to 50 µl of a mixture of [^{3}H]-E_1-S and unlabeled E_1-S (50 µM) and prewarmed to 37°C for 2–3 min. The reaction was started by the addition of 50 µl of the cell extracts. After incubation for 60 min, the reaction was stopped by addition of 2 ml of ethyl acetate and 200 µl of ice-cold water. The E_1 formed was extracted into the organic phase by shaking vigorously for 1 min. After centrifugation, 500 µl of the organic phase was removed and counted in a scintillation counter in 4 ml of liquid scintillant. A reference tube, containing 50 µl of the hot/cold estrone sulfate mixture, was also counted. Blanks consisted of assay tubes as described above, but the cell extract was added only after the reaction had been stopped. Specific activity was corrected for background activity and was expressed as pmol/min/mg protein.

Data Analysis

For each time point presented in this study, three separate incubations were performed in two independent cultures. Differences in enzymatic activity during osteoblast differentiation were evaluated by Student's *t*-test.

RESULTS

Cell Growth and Differentiation

Culture of SV-HFO cells in the presence of dexamethasone resulted in differentiation of

the cells, characterized by extracellular matrix synthesis and finally mineralisation. Figure 1 demonstrates collagen type I mRNA expression, alkaline phosphatase (ALP) expression, and calcium deposition in relation to culture time.

Analysis of Aromatase, 17β-HSD, and Sulfatase mRNA

At the indicated days, RNA was isolated and the presence of mRNA encoding the various enzymes was assessed via RT-PCR. PCR amplification with specific primers resulted in products of 372- and 240-bp coding for aromatase and sulfatase, respectively (Fig. 2). Digestion of the sulfatase product with *Kpn*I and *Dde*II gave fragments of the predicted sizes. Furthermore, the identity of the products was confirmed by hybridisation with a cDNA probe, specific for either aromatase or sulfatase (data not shown). The amounts of the PCR products increased linearly as a function of the amount of total RNA used. A mock RT reaction that contained all agents except the enzyme reverse transcriptase demonstrated that the amplified products were mRNA specific. Both aromatase and sulfatase mRNA were present in human osteoblasts. Semiquantitative analysis of the PCR products (normalised for 28S RNA expression) suggested that aromatase and sulfatase mRNA expression did not change during osteoblast differentiation (Fig. 2A). Transcripts for type 2, 3, and 4 isozymes of 17β-HSD were detected using RT-PCR, whereas type 1 expression was absent (Fig. 2B). Semiquantitative interpretation of the PCR products (normalised for 18S RNA expression) suggested a decrease in 17β-HSD type 2 and 3 expression and a slight increase in type 4 expression during osteoblast differentiation.

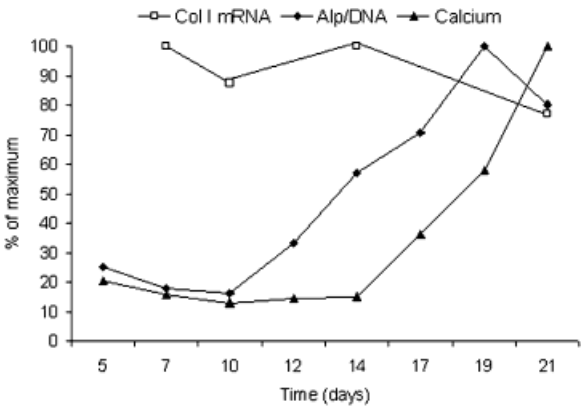

Fig. 1. Expression of collagen type I mRNA (Col I), alkaline phosphatase activity (ALP), and calcium deposition into the extracellular matrix during culture of SV-HFO cells. Cells were cultured and the various parameters determined as described under Materials and Methods. Data are expressed as percentage of maximum value during the culture for the specific characteristics.

Analysis of Aromatase and 17β-HSD Activity

Figure 3 depicts the conversion of A to E_1 and of T to E_2 in intact SV-HFO cells, demonstrating the presence of the enzyme aromatase. Aromatase activity was highest at day 6 but gradually decreased with increasing culture time (2.3-

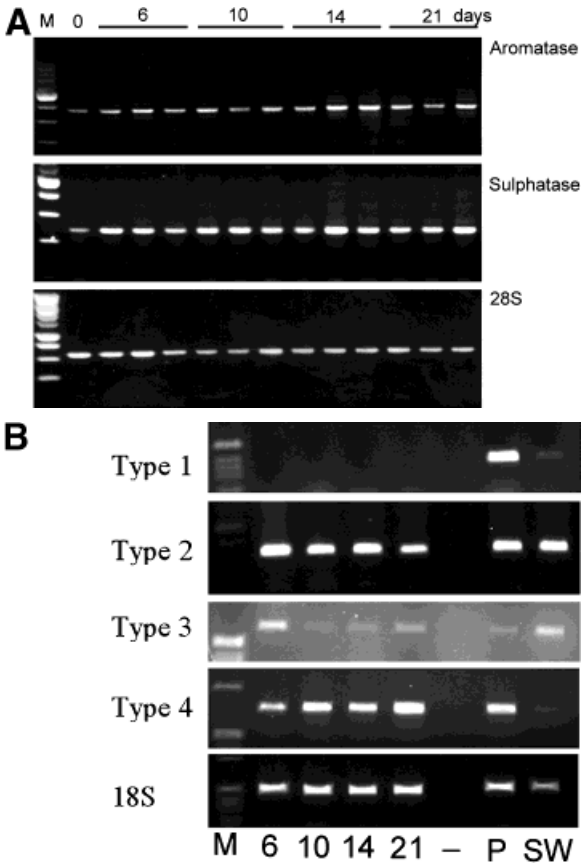

Fig. 2. Expression of **(A)** aromatase and sulfatase and **(B)** 17β-HSD isoforms mRNA during differentiation of SV-HFO cells. Cells were cultured as described under Materials and Methods; RNA was isolated on the days indicated. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to detect expression of aromatase, sulfatase, 17β-HSD type 1, 17β-HSD type 2, 17β-HSD type 3, 17β-HSD type 4, 28S, and 18S RNA. **A:** M, marker lane; 0, 6, 10, 14, and 21 days indicate the culture period and the day at which the RNA was isolated. **B:** M, marker lane; 6, 10, 14, and 21 indicate the culture period and the day at which the RNA was isolated; −, negative control; P, placenta (positive control for type 1, 2, 4); SW, SW620 cells (positive for type 3).

to 2.5-fold decrease at day 21 compared to day 6). T was aromatised to a lesser extent than A, suggesting a preference of aromatase for the substrate A. Incubations with A also resulted in the formation of E_2 (and small amounts of T) (data not shown). Likewise, incubations with T also yielded E_2 (and small amounts of A) as a product (data not shown). These data indicated the presence of both aromatase and 17β-HSD activity in SV-HFO cells, with the former predominating. To analyze this metabolism further, additional incubations were carried out using E_1 and E_2 as substrates.

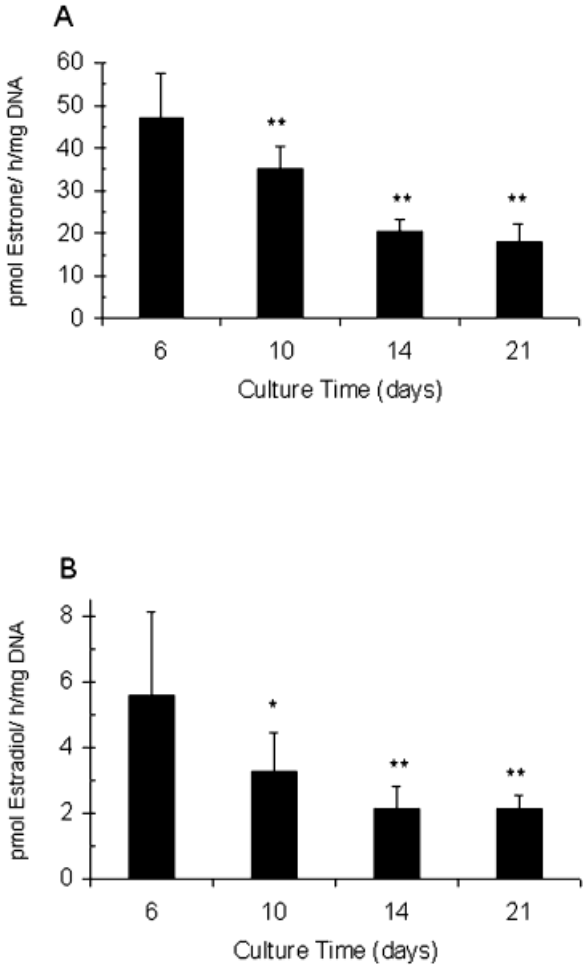

Fig. 3. Aromatase activity during osteoblast differentiation. Aromatase activity was assessed by incubating the cells at the indicated days with **(A)** androstenedione and **(B)** testosterone and the amounts of estrone and estradiol formed, respectively, were determined. The conversion of androstenedione and testosterone was linear up to 24-h substrate incubation. Data are expressed as pmol estrone/estradiol formed/h/mg DNA and are the means ±SD of the 4-, 8-, and 24-h substrate incubations, each consisting of duplicate wells from two independent experiments. $*P < 0.005$; $**P < 0.0005$ versus day 6.

The data shown in Figure 4A,B demonstrate both the conversion of E_1 to E_2 (i.e., reductive activity of 17β-HSD) and of E_2 to E_1 (i.e., oxidative activity). During differentiation, a progressive decline in reductive activity was observed, whereas oxidative activity only diminished from day 6 to 10 and then remained almost constant. Throughout the culture period, oxidative inactivation of E_2 predominated over the reductive reaction, which was even enhanced during differentiation (Fig. 4C). Conversion of A to T and T to A (also 17β-HSD reductive and oxidative activity, respectively) displayed the same pattern of changes in 17β-HSD activity (data not shown).

Analysis of Sulfatase Activity

Sulfatase activity was first examined in extracts of SV-HFO cells. Figure 5A depicts the amount of E_1 formed by conversion of E1-S, demonstrating the presence of sulfatase activity in cell extracts. During differentiation (i.e., with increasing culture time), no changes in sulfatase activity were observed. Next, in order to assess the physiological significance of sulfatase activity, experiments with intact cells were performed. Also, in intact cells, E_1 formation was observed, demonstrating that entrance of E_1-S into the osteoblasts is possible.

DISCUSSION

This study demonstrates that all enzymes involved in the formation of estradiol are present in human osteoblasts. These data support previous studies using rat and human osteosarcoma cells and human osteoblast-like cells, which indicated that estrogen metabolism in osteoblasts involves the coordinated action of aromatase and 17β-HSD isoenzymes [Eyre et al., 1998; Dong et al., 1998]. Furthermore, this study demonstrates for the first time that the activity of these enzymes changed with differentiation. Using A as the substrate, the predominant conversion throughout osteoblasts differentiation was aromatisation to E_1, although this activity decreased significantly by day 10 of culture (Fig. 3A). Similar observations were also made using T as the substrate for aromatase (Fig. 3B). We were unable to correlate this change in activity with a decreased expression of aromatase mRNA using semiquantitative RT-PCR. Aromatase produces a single protein, but the aromatase gene has alternative splicing sites upstream of the translation start

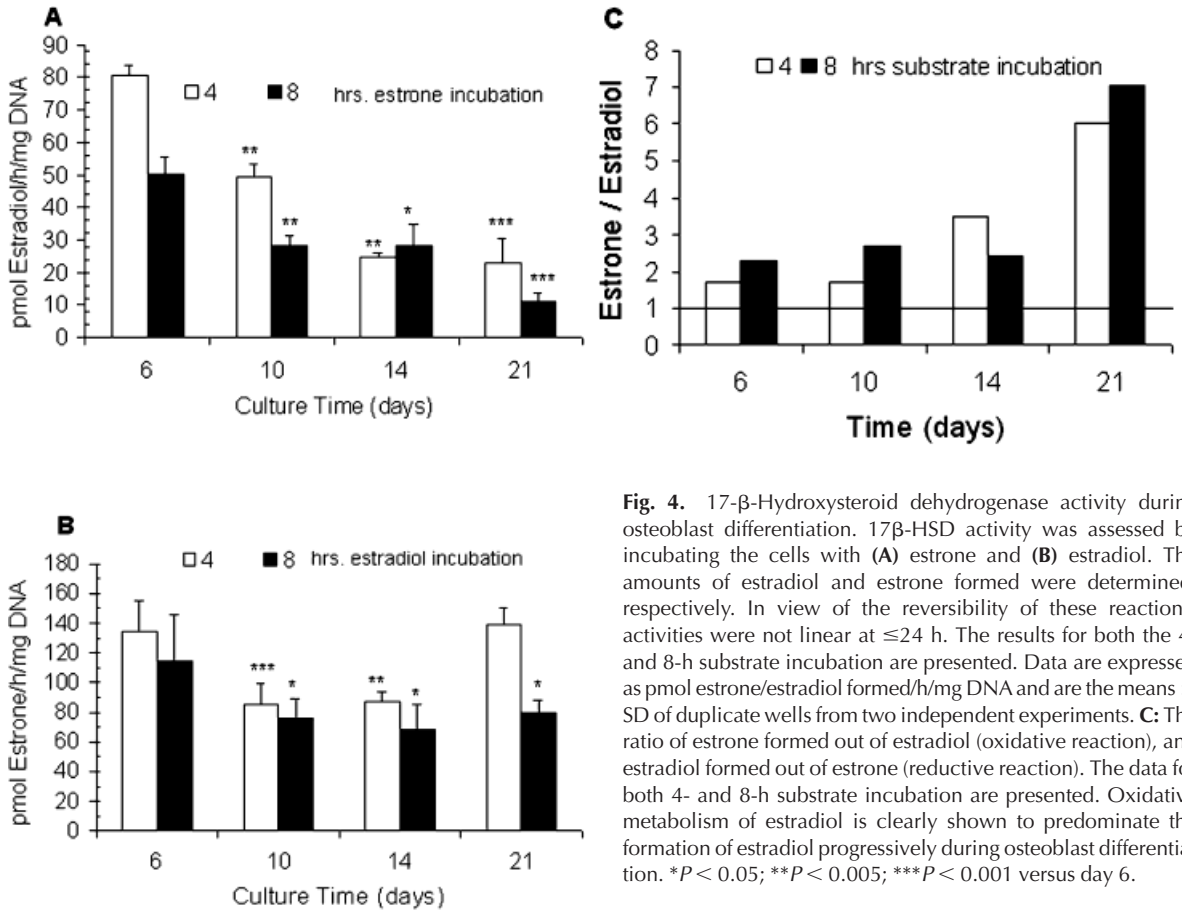

Fig. 4. 17-β-Hydroxysteroid dehydrogenase activity during osteoblast differentiation. 17β-HSD activity was assessed by incubating the cells with **(A)** estrone and **(B)** estradiol. The amounts of estradiol and estrone formed were determined, respectively. In view of the reversibility of these reactions, activities were not linear at ≤24 h. The results for both the 4- and 8-h substrate incubation are presented. Data are expressed as pmol estrone/estradiol formed/h/mg DNA and are the means ± SD of duplicate wells from two independent experiments. **C:** The ratio of estrone formed out of estradiol (oxidative reaction), and estradiol formed out of estrone (reductive reaction). The data for both 4- and 8-h substrate incubation are presented. Oxidative metabolism of estradiol is clearly shown to predominate the formation of estradiol progressively during osteoblast differentiation. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$ versus day 6.

site. These sites produce several promoter- and tissue-specific mRNA species corresponding to the untranslated 5′ exons [Toda et al., 1994]. The amplified fragment of aromatase cDNA corresponded to a region of the gene, which was 3′ of the ATG site in exon II. This approach enabled us to assess the overall presence of aromatase transcripts in SV-HFO but did not reflect changes in expression of promoter-specific aromatase mRNA transcripts. It is not likely, however, that the changes in aromatase activity, which we observed during differentiation, are attributable to promoter-specific transcripts, as alterations in gene expression would be reflected by the level of mRNA expression. Post-translational modulations, protein stability, or cofactor variations may be responsible for the observed changes in aromatase activity.

Although most studies of aromatase activity to date have used A as substrate, it is important to emphasise that this enzyme can also metabolize T to E_2. It was therefore interesting to note that SV-HFO cells produced both E_2 and A when T was used as a substrate. This observation highlights the significant role of 17β-HSD in controlling estrogen metabolism in osteoblasts. Further studies using E_1 and E_2 as substrates indicated that the human osteoblasts were able to carry out both oxidative (e.g. E_2 to E_1) and reductive (e.g., E_1 to E_2) reactions. As seen with aromatase, 17β-HSD reductive activity declined with differentiation. It would therefore appear that endogenous estradiol synthesis is more important during the early proliferative phase of osteoblast development than during a more differentiated mineralizing phase. It is interesting to compare these findings with those of previous studies using semiquantitative RT-PCR, which have suggested that aromatase mRNA is undetectable in normal bone or bone marrow, but expression is induced under pathological conditions such as fracture [Lea et al., 1997]. In contrast to 17β-HSD reductive activity, 17β-HSD oxidative activity only marginally changed during differentiation. At any phase of differentiation, the oxidative reaction was stronger than the reductive reaction (Fig. 4C). This finding suggests a

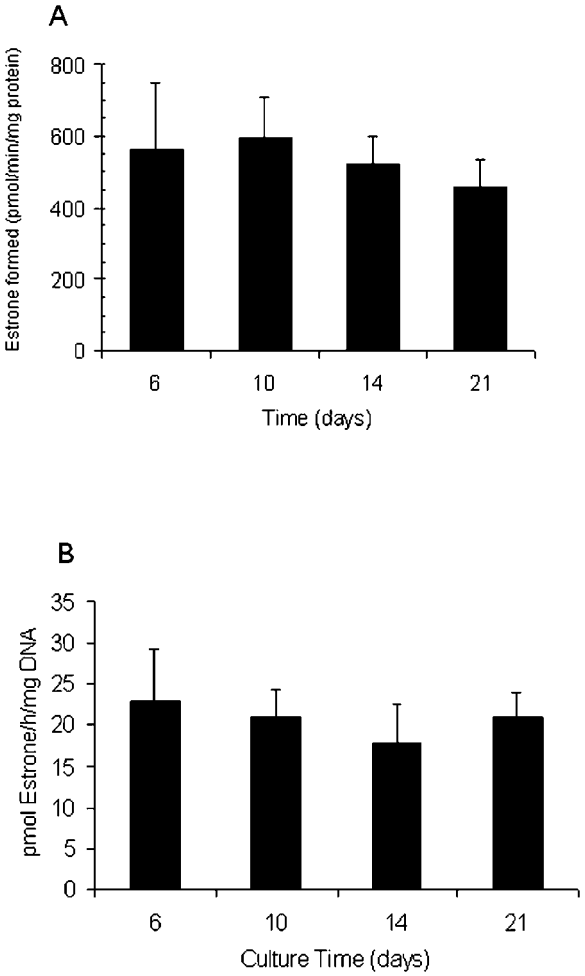

Fig. 5. Sulfatase activity in cell extracts and intact cells during osteoblast differentiation. At the days indicated, cell extracts were made and incubated with estrone-sulfate **(A)**, or intact cells were incubated with estrone-sulfate **(B).** Culture of the cells, preparation of the cell extracts, and sulfatase activity measurements are described under Materials and Methods. Data are presented as means ±SD of at least two independent experiments of each three separate cultures for the cell extract analysis and for the intact cell analysis duplicate wells from two independent experiments.

mechanism in osteoblasts that enables them to limit the effects of locally (and systemically) produced E_2 by converting it into E_1.

Changes in the estrogen endocrine system in human osteoblasts are not limited to the enzymes involved in the synthesis and metabolism of estradiol. Recently, we have shown that, depending on the differentiation status of the osteoblast, there is a subtype (α and β)-specific change in expression of the estrogen receptor [Arts et al., 1997]. However, the estrogen receptor expression appeared to be inversely related to the changes in aromatase activity and 17β-HSD conversion of E_1 into E_2. Whether this inverse relationship is related to a negative feedback control of E_2 on its synthesis in human osteoblasts or to the eventual consequences for osteoblast function is unknown. In the ovary an inverse relationship between estradiol formation and ER expression has been described after preovulatory follicular maturation induced by pregnant mare serum gonadotropin [Tetsuka et al., 1998]. It is speculated that this inverse relationship constitutes a compensatory mechanism to protect maturing follicles from over stimulation by estrogen. Whether a comparable mechanism is functional in osteoblasts is purely speculative. However, it can be concluded that the estrogen endocrine system in bone is dependent on osteoblast differentiation and that both metabolism, as well as receptor regulation, are involved.

The enzymatic activity data presented suggest that the interconversion of estrogens in SV-HFO cells involves more than one 17β-HSD isozyme. At least six isoforms of 17β-HSD have been cloned and characterised thus far. The sequence diversity and substrate specificity of these isoenzymes suggest that they have discrete roles within specific target tissues. Ovarian production of E_2 is catalyzed by the NADPH-dependent enzyme, 17β-HSD 1, which is also expressed in the mammary gland [Peltoketo et al., 1996]. The predominant activity of 17β-HSD 1 is reductive, preferentially metabolizing E_1 to E_2. NAD-dependent 17β-HSD 2 is also found in placental and ovarian tissue [Wu et al., 1993]. The enzyme carries out the reverse reaction to 17β-HSD 1, oxidizing E_2 to E_1, but it can also efficiently use T and dihydrotestosterone (DHT) as substrates. Type 3 17β-HSD is NADPH dependent and demonstrates predominant reductive activity. Until recently, the enzyme appeared to be found only in testis, preferentially catalyzing the conversion of androstenedione to testosterone [Geissler et al., 1994]. All three of these enzymes are members of the short-chain alcohol dehydrogenase (SCAD) family. SCAD function is also demonstrated by the ubiquitous NAD-dependent peroxisomal 17β-HSD 4 [Adamski et al., 1995] and the murine aldoketoreductase-like enzyme 17β-HSD 5 [Deyashiki et al., 1995]. The recently cloned 17β-HSD 6 was isolated from human prostate and has considerable sequence similarity to 3β-hydroxysteroid dehydrogenases (3β-HSDs) and retinol dehydrogenase type 1 [Biswas and Russell, 1997]. In a similar fashion, Penning and colleagues have

described a type 2 isoform of 3β-HSD that has bifunctional 3β- and 17β-HSD activity [Lin et al., 1998]. RT-PCR analysis of 17β-HSD mRNA expression revealed transcripts for isozymes 2, 3, and 4, with only very weak expression of 17β-HSD 1. It is interesting to note that we have previously reported a similar pattern of 17β-HSD isozyme expression in rat osteosarcoma cells, although in this case the predominant activity was clearly reductive [Eyre et al., 1998]. Our data do not give any clear indication as to which 17β-HSD isozyme(s) is the target for regulation during OB cell development. The most obvious candidate for the reductive activity observed in SV-HFO cells is 17β-HSD-3, as these cells appear to express relatively low amounts of 17β-HSD 1. However, only very low levels of A to T conversion (the preferred reaction for 17β-HSD 3) were observed in SV-HFO cells, and this was unaffected by cell differentiation. It is possible that reductive estrogen metabolism observed in SV-HFO cells is the result of low-affinity conversion by the predominantly oxidative type 2 or type 4 isozymes, or both.

The present study also demonstrates the presence of sulfatase activity in SV-HFO cells and in cell extracts and thereby supports previous findings obtained with human osteosarcoma cells [Fujikawa et al., 1997]. In contrast to aromatase and 17β-HSD activity, sulfatase expression and activity are independent of osteoblast differentiation. Although declining with age, the still considerable amounts of DHEA-S and E_1-S circulating after menopause suggest that the sulfatase enzyme system is of importance. The extent to which it contributes to estradiol formation as compared with the aromatase 17β-HSD system remains unclear. It seems likely that the sulfatase system serves as a general supply system of estradiol precursors, while the aromatase 17β-HSD system is involved in control of estradiol levels and estradiol specific effects at cellular level.

In conclusion, the human SV-HFO osteoblasts contain the enzymes, aromatase, 17β-HSD, and sulfatase, involved in estradiol synthesis and activity of some of these enzymes is related to the differentiation of the cells. The capacity of osteoblasts to synthesize and convert estradiol may influence the rate of bone turnover, and thereby postmenopausal bone loss. It is interesting to speculate on the possibilities of intervention in osteoporosis by modulating specific enzymatic activities. It would be particularly interesting to assess the identity and regulation of the 17β-HSD isoforms involved in controlling the balance between oxidative or reductive metabolism, because inhibition or stimulation might lead to an increase in estradiol concentration, which in turn could affect bone turnover.

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