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Steroid Sulfatase Activity in Osteoblast Cells

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We have demonstrated steroid sulfatase activity in osteoblast cells and characteristics of the enzyme were also investigated. Cell free homogenate of rat osteoblast cell line, UMR106-01 and human osteoblast cell lines, MG-63, HOS were incubated with [^{3}H] dehydroepiandrosterone-sulfate (DHEA-sulfate) or [^{3}H] estrone-sulfate (E_1-sulfate). The formation of DHEA or E_1 from the corresponding substrate was identified by crystallization to constant specific activity. Michaelis constant (Km) for DHEA-sulfate was estimated as 2.1×10^{-8}M in UMR106-01, 7.4×10^{-7}M in MG-63, 5.8×10^{-7}M in HOS and that for E_1-sulfate was 4.1×10^{-7}M, 3.0×10^{-7}M, 9.8×10^{-7}M, respectively. The expression of steroid sulfatase messenger ribonucleic acid in human osteoblast cells, HOS and MG-63 was first demonstrated by reverse transcription-polymerase chain reaction. The existence of steroid sulfatase in human and rat osteoblast cells suggests that osteoblast cells have the capacity to convert circulating sulfo-conjugated steroids to more active androgens and estrogens. This may indicate an important role of bone in facilitating hormonal action.

Since estrogen and androgen receptors were found in osteoblast cells [1-3], a direct effect of sex steroids on the control of cell growth and the proliferation in osteoblasts is suggested. In clinical situations, menopause has been implicated as a factor in the development of osteoporosis. Estrogens have long been noted to exert profound effects on indices of bone and mineral metabolism as well as on bone mass [4-8]. In addition, it is reported that androgen insufficiency in men is clearly related to the development of osteopenia [9-11] and androgen replacement appears to partially reverse the osteopenia present in hypogonadal men [12]. Therefore, estrogens and androgens are important factors in bone and mineral metabolism.

Since enzyme systems including aromatase, 5α-reductase and 17β-hydroxysteroid dehydrogenase (17β-HSD) activities were found in osteoblast cells [13-16], the possibility that osteoblast cells may control their own cell growth and proliferation by producing biologically active steroids is suggested. Purohit et al. [13] reported that estrone (E_1) was synthesized from both [^{3}H] E_1-sulfate and androstenedione in osteoblast cells. However, they did not mention the availability of sulpho-conjugated androgen as the substrate of steroid sulfatase in osteoblast cells. It is known that conjugated steroids such as dehydroepiandrosterone-sulfate (DHEA-sulfate) are abundant in peripheral circulations and are biologically less active than the corresponding unconjugated steroids. To determine whether osteoblast cells can hydrolyze circulating conjugated steroids and convert them to biologically active androgens and estrogens, we examined the existence and characteristics of steroid sulfatase (E.C.3.1.6.2.) in osteoblast cells.

MATERIALS AND METHODS

Materials. Eagle's minimal essential medium (MEM) and fetal calf serum (FCS) were purchased from GIBCO (Grand Island, NY). [1,2,6,7-^{3}H] DHA-sulfate (3.4TBq/mmol) and [6,7-^{3}H] E_1-sulfate (1.6TBq/mmol) were purchased from New England Nuclear Corp. (Boston, MA). Unlabeled steroids were purchased from Sigma Chemical Co. (ST.Louis, MO). Thin-layer chromatography (silica gel 60F, Merck, Darmstadt, Germany) was used to separate steroids. All other chemicals were of reagent grade and obtained commercially.

Cell culture. Osteoblast cell lines, UMR106-01 from rat osteosarcoma, and HOS and MG-63 from human osteosarcoma, were purchased from ATCC (Rockville, MD). Cells were cultured in 10ml tissue culture dishes (Nunc, Denmark) in MEM with Earle's salts and 20mM Hepes buffer. All media were supplemented with 2 mM L-glutamine, 10 mM sodium hydrogen carbonate, 1% nonessential amino acids and 10% FCS. For the measurement of steroid sulfatase activity, UMR106-01, MG-63 and HOS cells were used. When the cells reached confluence, they were rinsed three times with phosphate buffered saline (PBS) and were scraped from the culture dishes

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and then homogenized with ice cold PBS by an ultrasonic homogenizer for 20 sec. Sulfatase activity was measured in the cell free homogenate.

For the determination of messenger ribonucleic acid (mRNA) levels of steroid sulfatase in osteoblast cells, MG-63 and HOS cells were used. After removal of media, culture dishes were kept frozen at −80°C until analysis.

Measurement of steroid sulfatase activity. The formation of DHEA from [^{3}H] DHEA-sulfate and E_1 from [^{3}H] E_1-sulfate in UMR106-01, MG-63 and HOS were determined by in vitro incubation [17,18]. [^{3}H] DHEA-sulfate (200,000 dpm, 22.3nM) or [^{3}H] E_1-sulfate (200,000 dpm, 16.3 nM) was dissolved in 2 drops of ethylene glycol and the enzyme suspension (0.15 ml) was added to the medium (10 mM PBS, pH 7.4, total volume; 1ml) and incubated at 37°C for 60 min. Incubation was terminated by the addition of chilled ethyl acetate (4 ml) and 2,000 dpm of ^{14}C-DHEA or ^{14}C-E_1 was added to correct for recovery losses and for identification of products. The organic phase was separated by centrifugation and steroids were extracted with ethyl acetate. Radiolabeled products were separated by thin-layer chromatography (cyclohexane : ethyl acetate = 1 : 1 v/v). The silica gel areas corresponding to authentic conjugated and unconjugated steroids were removed by scraping and radioactivity was assayed using a liquid scintillation counter (Aloca LSC-651, Tokyo). All incubations were performed in duplicate. Final identification was accomplished by recrystallization to constant specific activity with authentic standard. ^{3}H/^{14}C ratios were also calculated.

Protein concentrations were estimated with a commercial dye reagent (ToneinTP kit, Otsuka Pharmaceuticals, Tokyo) according to the method of Bradford [19].

Determination of mRNA levels by reverse transcription-polymerase chain reaction (RT-PCR) in HOS and MG-63. Three ml of Isogen (Nippon Gene Co., Kanazawa) was added to the frozen culture dish and total RNA was isolated from the cells by the method of Chomcynski and Sacchi [20]. Steroid sulfatase-cDNA was synthesized by the RNA-PCR kit (Perkin-Elmer, Branchburg, NJ). One μg aliquots of total RNA extracted were added to 19 μl of reverse transcriptase reaction mixture for a final concentration of 10mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, dNTPs (each at 1 mM). 1 U/μl RNase inhibitor, 16 U/μl oligo d(T) and 2.5 U/μl of Murine Leukemia Virus reverse transcriptase were added to the mixture in a final volume of 20 μl at room temperature, and further incubated at 42°C for 15 min, 99°C for 5 min and 5°C for 5 min [21].

PCR procedure and product analysis were performed as previously described [22]. One μl aliquots of the cDNA were added to 24 μl of PCR mixes for a final concentration of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 0.001% (w/v) geratin, 0.25 μl of each dNTP, 0.25 mM of each primer and 1.25 units of Taq polymerase in a total volume of 25 μl. The sequence of sense and antisense primers to amplify the open reading frame of cDNA were 5′-GAACACTGAGACTCCGTTCCT-3′ (exon 6) and 5′-CTTTATAGATCCCATTACTTCCGCC (exon 7), respectively [22,23].

Samples were covered with 30 μl mineral oil and followed by 30 cycles of PCR. One PCR cycle consists of denaturation at 94°C for 30 sec, annealing at 59°C for 30 sec and polymerization at 72°C for 2 min. The expected size of the PCR products for steroid sulfatase are 275bp. PCR products were electrophoresed on a 2.5% agarose gel in 0.5 × TBE, visualized by ethidium bromide (0.5 μg/ml gel) staining and photographed using an ultraviolet transilluminater. For the positive control, same procedure was performed using human term placenta.

RESULTS

Measurement of steroid sulfatase activity. [^{3}H] DHEA-sulfate and [^{3}H] E_1-sulfate were incubated with the cell free homogenate of UMR 106-01 for 60 min. The radiolabeled products were developed on TLC and radioactivity in the areas corresponding to DHEA and E_1 areas was measured. The formation of DHEA from [^{3}H] DHEA-sulfate was 0.4 pmol/mg protein/hr and E_1 from E_1-sulfate was 0.3 pmol/mg protein/hr. The radiochemical purities of the isolated products, DHEA and E_1, were established by demonstrating constant specific radioactivity of the ^{3}H/^{14}C ratio through repeated recrystallization with non labeled authentic standard (Table 1). The specific activities of the three crystals were successive crystallizations by less than 5%. When the duration of incubation or the protein concentration was varied, the rate of DHEA-sulfate hydrolysis had a linear relationship to incubation time up to 2 hr and to protein concentration up to 0.4mg protein per incubation tube (Fig. 1). A similar relationship between the duration of incubation or protein concentration and the amount of product was observed when E_1-sulfate was used as a substrate (Fig. 2). The apparent Km of steroid sulfatase for DHEA-sulfate in UMR106-01 was estimated as 2.1×10^{-8}M with a maximum velocity (Vmax) of 50 pmol/mg protein/hr by the method of Lineweaver-Burk (Fig. 3). The Km value for E_1-sulfate and its Vmax were found to be and 4.1×10^{-7}M, 47 pmol/mg protein/hr. In MG-63 Km and Vmax for DHEA-sulfate were 7.4×10^{-7}M, 12.7 pmol/mg protein/hr, E_1-sulfate were 3.0×10^{-7}M, 0.50 nmol/mg protein/hr (Fig. 4). In HOS, those were 5.8×10^{-7}M, 1.47 pmol/mg protein/hr, 1.7×10^{-7}M, 0.19 nmol/mg protein/hr, respectively (Fig. 5).

Detection of steroid sulfatase mRNA in HOS and MG-63 by RT-PCR. Total RNA was extracted from HOS

TABLE 1

Radiochemical Purities of Labeled Steroids

			Crystallization			
Substrates	Metabolites	Original	X-1	X-2	X-3	ML-3
DHEA-sulfate	DHEA	3.429	2.453	2.564	2.531	2.472
E1-sulfate	E1	7.702	7.045	7.241	7.085	7.664
Solvent used			n-heptane benzene	n-hexane methanol	n-heptane methanol	

Figures are expressed as ^{3}H/^{14}C ratio.

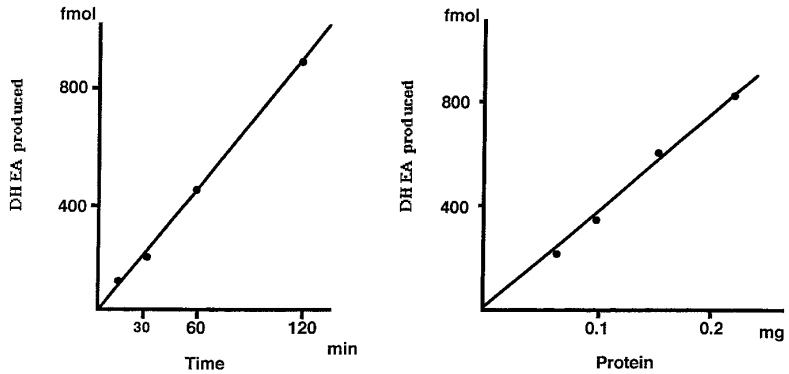

FIG. 1. Formation of DHEA from DHEA-sulfate in UMR106-01. In the left panel, the incubation time was varied and 0.2 mg protein was used in the experiment. In the right panel, the amount of protein was varied and incubation was continued for 1 hour. The other experimental conditions are described in the text.

and MG-63 cells, and cDNA was transcribed by the RNA-PCR kit. Thirty cycles of PCR were carried out to produce human steroid sulfatase cDNA by these cDNA and sense and antisense primers. The PCR product was electrophoresed and mRNA of steroid sulfatase was detected in human osteoblast cells HOS and MG-63 (Fig. 6). When aliquots of total RNA extracts from MG-63 and HOS were processed to PCR step without reverse transcriptase reaction, no PCR product was detected. The expression placental mRNA of steroid sulfatase was shown in Fig. 6-lane 5 as a positive control.

DISCUSSION

In the present study, the kinetics of steroid sulfatase in rat and human osteoblast like cell line firstly was demonstrated. And the expression of steroid sulfatase mRNA was also firstly detected in human osteoblast cells. These results indicate that osteoblasts have the capacity to convert sulfo-conjugated steroids, such as DHEA-sulfate and E_1-sulfate into more biologically potent androgens and estrogens.

Osteoblastic functions may be influenced not only by estrogen and androgen via receptor-mediated process but also by the local formation of biologically potent steroids in the cells. Aromatase, estrone sulfatase, 5α-reductase and 17β-HSD activities in osteoblast cells have been reported [13-16]. Aromatase in osteoblast cells may contribute to the osteoblastic function by the formation of estrogen from androgen. Estrone sulfatase, 5α-reductase and 17β-HSD activities may also exert an effect on the function of osteoblasts by converting inactive steroids to active forms.

Steroid sulfatase activity is found in various tissues, including placenta, skin, liver, gonadal tissues, breast cancer and endometrial cancer cells. Evidence has been presented that arylsulfatase C and steroid sulfatase is ranked among the microsomal type I arylsulfatases. However, it is not yet clear whether this group really consists of several different enzyme species or of only one sulfatase of low substrate specificity [24]. E_1-sulfate is known to be a principal estrogen in postmenopausal women. Purohit et al. reported the formation of E_1 from [^{3}H] E_1-sulfate by whole cell assay of human

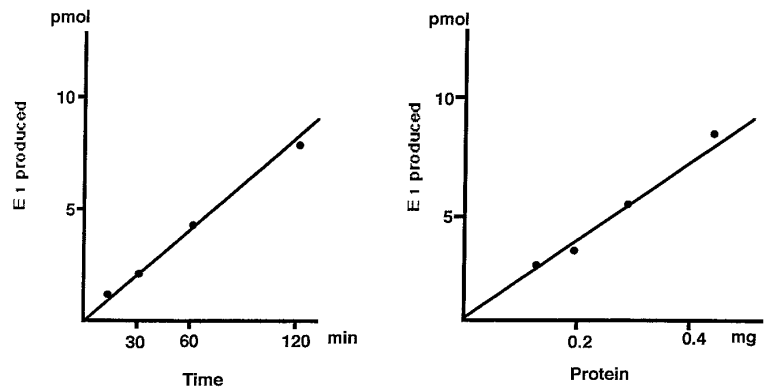

FIG. 2. Formation of E_1 from E_1-sulfate in UMR106-01. In the left panel, the incubation time was varied and 0.2 mg protein was used in the experiment. In the right panel, the amount of protein was varied and incubation was continued for 1 hour.

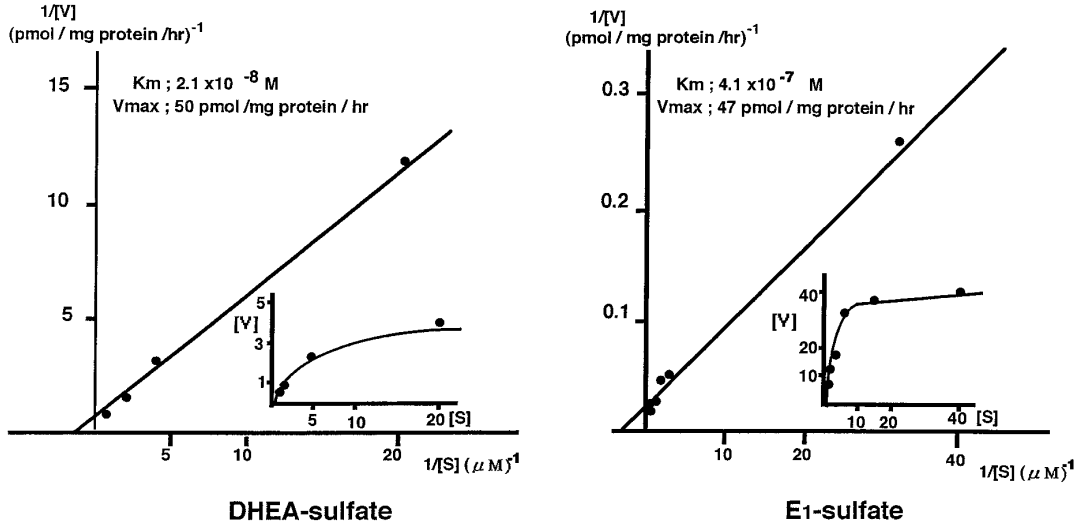

FIG. 3. The effect of DHEA-sulfate (left) and E_1-sulfate (right) concentration on sulfatase activity in UMR106-01. Incubation was continued for 1 hour and 0.2 mg protein was used. Km value for DHEA-sulfate was estimated as 2.1×10^{-8}M with Vmax of 50 pmol/mg protein/hr by Lineweaver–Burk analysis. Km for E_1-sulfate was 4.1×10^{-7}M and Vmax was 47 pmol/mg protein/hr.

osteoblast cells HOS, MG-63 and U2OS [13]. They also speculated on the role of osteoblast cells in the bone metabolism by virtue of possessing several steroid bioconverting enzymes. But, they did not mention the substrate specificity and property of this enzyme in osteoblast cells.

Therefore, in the present study, the characteristics of steroid sulfatase activity were firstly demonstrated in UMR106-01 and MG-63, HOS by in vitro incubation using a cell free homogenate. The formation of E_1 from [^{3}H] E_1-sulfate and DHEA from [^{3}H] DHEA-sulfate was established by recrystallization. The Km values for DHEA-sulfate and E_1-sulfate in MG-63 and HOS were estimated from 1.7×10^{-7}M to 7.4×10^{-7}M. Although placental tissue is known to have the highest sulfatase activity, the Km values for DHEA-sulfate and E_1-sulfate in osteoblasts obtained from the present study are comparable to those found in human placenta [24]. The Vmax value for E_1-sulfate was higher than DHEA-sulfate in MG-63 and HOS. This result corresponded the

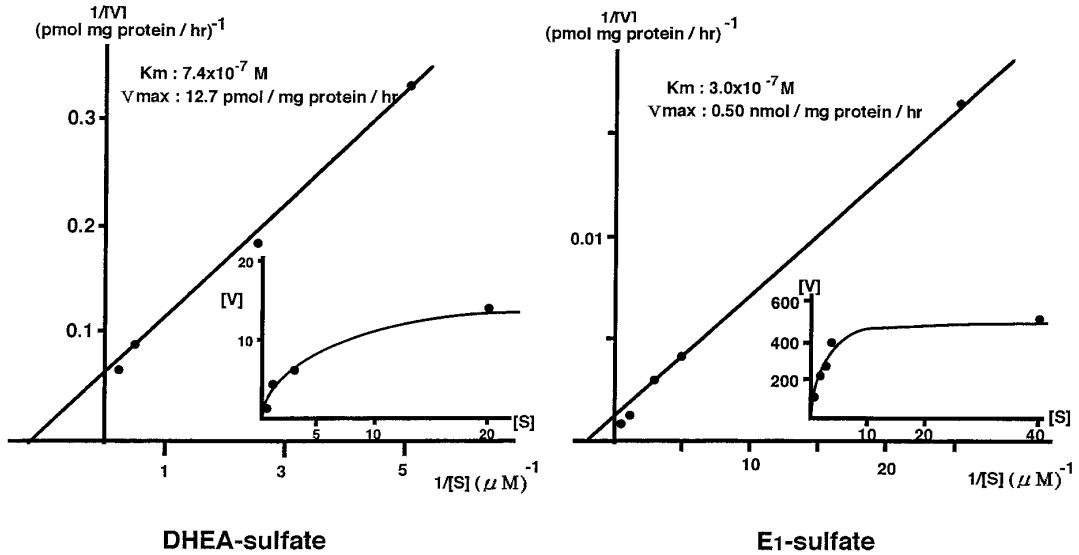

FIG. 4. The effect of DHEA-sulfate (left) and E_1-sulfate (right) concentration on sulfatase activity in MG-63. Incubation was continued for 1 hour and 0.2 mg protein was used. Km value for DHEA-sulfate was estimated as 7.4×10^{-7}M, with Vmax of 12.7 pmol/mg protein/hr by Lineweaver–Burk analysis. Km for E_1-sulfate was 3.0×10^{-7}M and Vmax was 0.50 nmol/mg protein/hr.

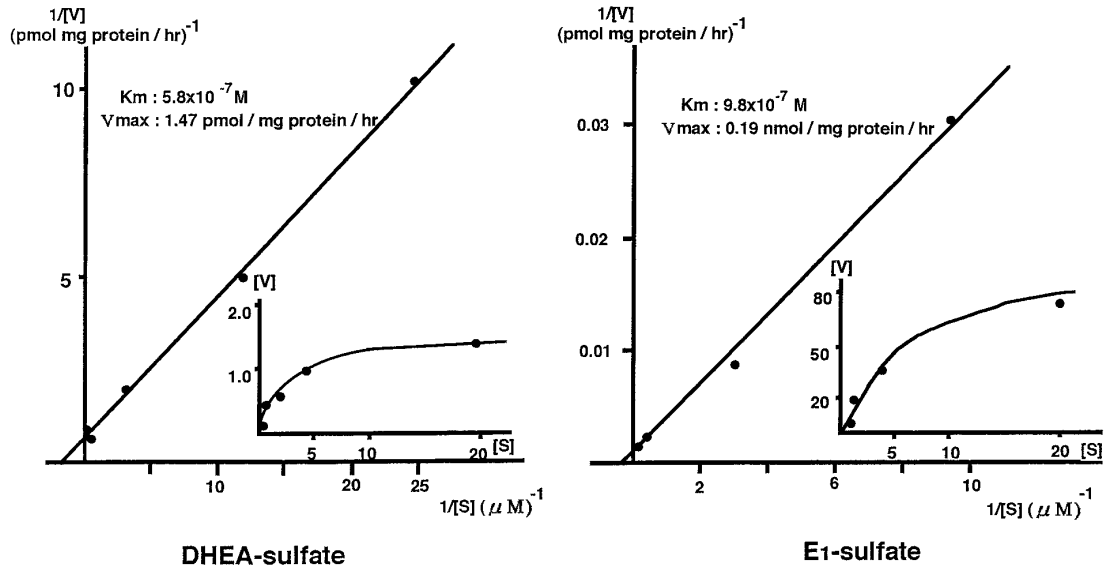

FIG. 5. The effect of DHEA-sulfate (left) and E_1-sulfate (right) concentration on sulfatase activity in HOS. Incubation was continued for 1 hour and 0.2 mg protein was used. Km value for DHEA-sulfate was estimated as 5.8×10^{-7}M with Vmax of 1.47 pmol/mg protein/hr by Lineweaver–Burk analysis. Km for E_1-sulfate was 9.8×10^{-7}M and Vmax was 0.19 pmol/mg protein/hr.

literature in human breast cancer [25] and human benign prostatic hyperplasia [26]. High affinity of steroid sulfatase for DHEA-sulfate was found in UMR106-01 and similar results were reported in rat brain steroid sulfatase [27].

Estrogen deficiency associated with menopause has been implicated as a factor in the development of osteoporosis. In the past few years increasing evidence has accumulated supporting an increase in bone absorbing cytokines as a possible mechanism for postmenopausal bone loss [28] and the interaction of osteoblast and osteoclast cells on bone metabolism is also suggested [29]. Androgen and estrogen inhibit interleukin-6 (IL-6) production by receptor mediated suppression of IL-6 promoter gene [30,31]. Due to the deficiency of estrogen during menopause, an increase in the production of IL-6 in bone marrow cells stimulates osteoclast development [28]. DHEA-sulfate, a major adrenocortical steroid, is the most abundant steroid in the peripheral circulation and its concentration gradually decreases with age [32]. Recently, in postmenopausal women, a positive correlation between bone mineral density and serum DHEA-sulfate level has been reported [14]. It is of interest that osteoblast cells possess the enzyme converting biologically less active DHEA-sulfate and E_1-sulfate to their active forms as shown in the present investigation. These studies imply that the decrement of circulating levels of biological active steroid precursors consequently result in the decrease of local concentration of the hormones, since sulfatase activity in the first step to form the active steroid from inactive sulfoconjugated steroid. These results suggest that this enzymatic activity may be an important component of osteoblastic function. Effect of aging on sulfatase activity in osteoblast cells as well as the control mechanism of the enzymatic activity have to be studied in the future.

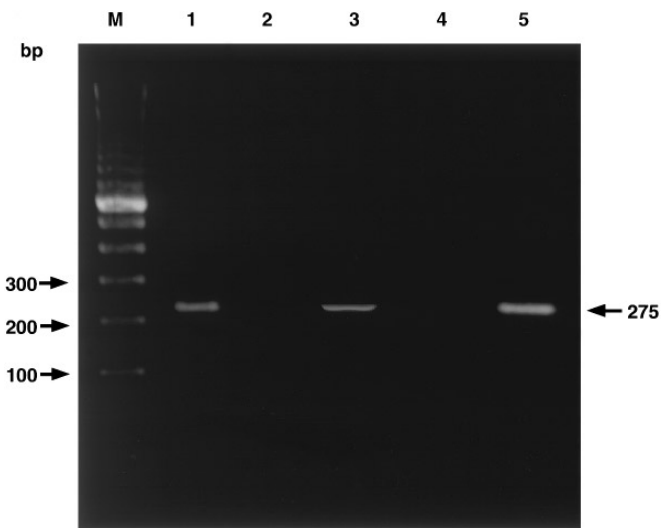

FIG. 6. Analysis of human steroid sulfatase mRNA. Total RNA from HOS, MG-63 and placenta tissue was reverse-transcribed to cDNA followed by amplification of exon 6-7 (275bp) of the sulfatase gene by PCR. PCR products were electrophoresed. M, molecular weight standard marker; lane 1, HOS; lane 2, negative control (HOS); lane 3, MG-63; lane 4, negative control (MG-63); lane 5, positive control (placenta homogenate).

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