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STIMULATORY EFFECTS OF ESTROGEN AND PROGESTERONE ON PROLIFERATION AND DIFFERENTIATION OF NORMAL HUMAN OSTEOBLAST-LIKE CELLS *IN VITRO*

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Here we report that osteoblast-like cells derived from female and male adult human trabecular bone are able to directly respond to 17β-estradiol (E2) and progesterone (P). In short-term (1 day) cultures using serum-free and phenol red-free medium, both steroid hormones were found to stimulate DNA synthesis and growth of the human osteoblast-like cells. P was more potent in stimulating osteoblast growth compared to E2. On the other hand, E2 showed a stronger differentiation-inducing effect as determined by analysis of the number of cells displaying cytochemical alkaline phosphatase (AP) activity, a marker for the mature osteoblast phenotype. Combination of E2 and P resulted in a further increase in DNA synthesis, but did not further affect the number of cells expressing AP activity. In conclusion, female sex steroids may be involved in regulating bone mass in human adults via a direct anabolic action on the bone forming cells.

Deficiency of estrogen and progesterone is believed to be one of the major causes of postmenopausal osteoporosis. Estrogen/progesterone substitution therapy has been shown to be effective of preventing excess bone loss, however, the precise mechanism of the action of these steroid hormones on bone metabolism is not yet fully clear (for reviews: 1-3). The demonstration of the presence of specific estrogen and (inducible) progesterone receptors in osteoblast-like cells (4,5) raised the possiblity of a direct anabolic action of these hormones on the bone forming cells, next to the assumption of an indirect skeletal effect via systemic, intermediate factors (1-3). *In vitro* studies have yielded both stimulatory as inhibitory effects by estrogen on osteoblastic cells from different sources (6-10). Recent reports using a culture system of normal human bone-derived cells have been unable to demonstrate

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significant effects of estrogen (11-12). Nonetheless, specific estrogen receptor binding has been demonstrated in these cells (5, 13) as well as enhanced pro-collagen type I mRNA levels (13) and proto-oncogene c-myc expression under the influence of estrogen (14). The present study was designed to explore and to compare estrogen and progesterone effects on the growth and differentiation of the normal human osteoblast-like cells derived from female or male trabecular bones.

MATERIALS AND METHODS

Human osteoblast (HOB) cultures. Adult human trabecular bone explants were dissected from femoral heads obtained during orthopaedic surgery. Osteoblast-like cell cultures were subsequently established from these bone particles as described previously (15,16). Culture was performed in 10% fetal bovine serum (FBS) in minimal essential medium (MEM) supplemented with 2mM glutamine, 0.1 mg/ml streptomycin, 100 U/ml penicillin, 2.5 μg/ml fungizone in 5% CO_2/air at 37 °C. The bone-derived cells display specific osteoblast features, such as alkaline phosphatase activity, production of osteocalcin and collagen type I and the ability of minerilized bone matrix formation during culture.
HOB from bone samples derived from different patients were harvested using 0.05% trypsin/0.02% EDTA treatment, and subsequently pooled before subculture in 96-well culture plates (5,000 cells per well). After 3-5 days preculture in MEM/FBS, medium was replaced with serum-free, phenol red-free MEM containing 1 mg/ml bovine serum albumin (BSA) and the supplements as above. After 4 hours, medium was refreshed with either control medium or medium containing 17β-estradiol (E2) or progesterone (Sigma). The following assays were carried out in the 96-well plates after incubation of the cultures for 1-2 days.

Total cell number assay. Total cell numbers were determined using methylene blue staining of fixed adherent cells as described previously (17,18). Cultures were fixed with neutral buffered formalin for 30 min at room temperature, stained with 0.1% methylene blue in 0.01 M borate buffer (pH 8.6) for 30 min, followed by a rinse procedure with borate buffer. Then the dye was eluted in a 1:1 ethanol (v/v 99.8%) and 0.1 M HCl solution, and the absorbance at 595 nm was measured using an automatic microtiter plate reader. Results were calculated relative to control absorbance values.

BrdU-incorporation. DNA synthesis of the cells was studied by immunochemical determination of the amount of 5-bromo-2'-deoxy-uridine (BrdU), a thymidine analogue, incorporated into cellular DNA (19). The cultures were labeled with 10 μM BrdU (Amersham) during the whole incubation period (24 h.), fixed in 70% ethanol for 30 min. at 4 °C, followed by a 15 min. treatment with 4 N HCl. After subsequent washing in 0.1 M borate solution (pH 8.5) and twice in PBS, the cells were overlayered with a specific mouse anti-BrdU monoclonal antibody (0.5 μg/ml; Boehringer-Mannheim) for 60 min. at room temperature. After reaction with goat anti-mouse IgG conjugated with peroxidase for 60 min., antibody labeling was detected using ABTS as substrate (Boehringer-Mannheim). The absorbance of the reaction was quantified with a microtiter plate reader at 414 nm. The results were expressed as a percentage of control values.

Analysis of alkaline phosphatase (AP) activity. Histochemical staining of the cells for AP activity was carried out using a Sigma kit (No. 84) with naphtol AS-BI phosphate as substrate and Fast Blue BB as coupler. The number of all detectable positive cells was counted in random microscopical fields using an inverted microscope. Biochemical AP analysis was detected on cells treated with 0.05% Triton-X for 5 min. followed by addition of 0.1 M aminoethanol solution (pH 10.5) containing 150 mM NaCl, 2 mM $MgCl_2$ and 2.5 mg/ml p-nitrophenyl phosphate. Production of p-nitrophenol after an incubation time of 2 hrs at 37 °C was quantified by measuring the absorbance at 405 nm using a microtiter plate reader.

Statistics. Results from several identical, but independent experiments (using different pooled cell populations) were collected and subjected to an overall statistical evaluation using the Student's t-test and the Student-Newman-Keul's Oneway Analysis of Variance.

RESULTS

As shown previously, the methylene blue staining (MB) assay is a suitable approach to analyse total number of human osteoblast-like cells (HOBs) and the effects of growth factors and hormones on osteoblast multiplication (18). The staining reaction as measured by the absorbance of the eluents gave a linear relation with cell numbers (Fig. 1). Fig. 2 depicts the overall results obtained from HOB cultures incubated with either E2 or progesterone in serum-free medium for one day. Both sex hormones significantly stimulated HOB cell proliferation. Progesterone was a more potent stimulator of HOB growth than E2. Co-variance analyses of the results comparing female and male cultures excluding group effects (different hormone treatments) revealed a significant sex-specific difference ($P<0.05$). The findings obtained with the MB assay were corroborated by direct microscopic cell countings (not shown) and BrdU-incorporation experiments assessing DNA synthesis of male-

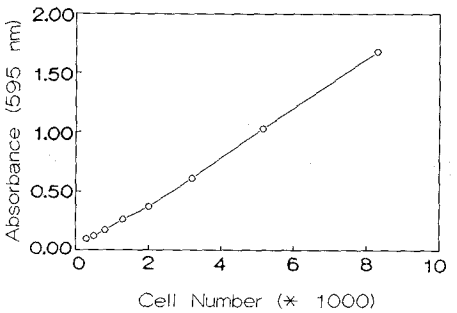
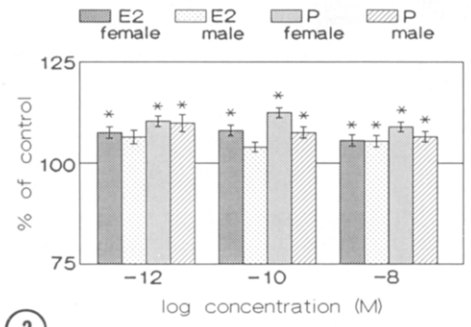


Fig. 1. Linear relationship between cell numbers and absorbance of dye eluted from HOB stained with methylene blue after culture in 96-well test plates.



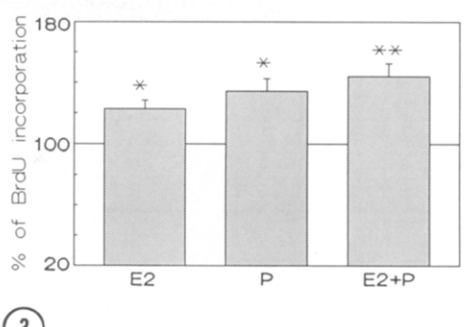
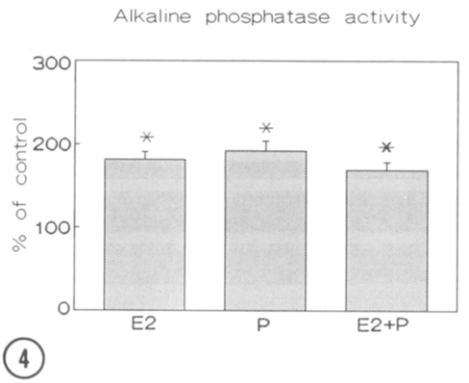


Fig. 2. Effect of E2 and progesterone on proliferation of HOB derived from either female or male trabecular bone samples. The cells were treated with the hormones for 1 day in serum-free, phenol-red medium. Results ± SEM are from 5-6 independent experiments using pooled HOB strains established from several individuals and are expressed as relative to control absorbance values using the methylene blue (total cell number) assay. * significantly different compared to control cultures ($P<0.05$).

Fig. 3. Effect of 10^{-10}M E2 and 10^{-10}M progesterone on BrdU incorporation (measure for DNA synthesis) into HOB derived from female operation material. Means ± SEM are expressed relative to control values (n=9-10; 2 experiments). * $P<0.05$ versus control; ** $P<0.01$ versus controls and cultures treated with E2 alone.

derived (not shown) and female-derived cells (Fig. 3). Combination of E2 and progesterone during culture resulted in an enhanced DNA synthesis of the HOBs (Fig. 3). In HOB cultures maintained for two days in serum-free media, however, the effects of the hormones were greatly diminished. Proliferation studies assessing DNA synthesis (results not shown) and total cell numbers did not yield significant effects in the two days' E2-cultures and reduced effects in the progesterone cultures (relative MB absorbance of 5 independent pooled experiments ± SD: 10^{-10}M E2; 104.4 ± 4.8 % ; 10^{-10}M progesterone: 107.5 ± 3.8, $P<0.05$).

The effects of the hormones on osteoblastic cell differentiation were studied using alkaline phosphatase (AP) as a marker for mature osteoblast phenotype (e.g. 19). Biochemical analysis revealed a significant and equal increase of the AP activity in the E2 and progesterone-treated cultures (Fig. 4). Combination of the steroids did not result in a further increment of the AP activty (Fig. 4). Bearing in mind the stronger proliferative effects by progesterone in these cultures (Figs. 2,3), it would imply that the number of cells expressing more intense AP activity should be higher in the E2 cultures. This was indeed confirmed by cytochemical analyses of the HOB cultures stained for AP activity as shown in Fig. 5, indicating that E2 preferentially promoted differentiation-inducing effects in the human bone cell cultures.



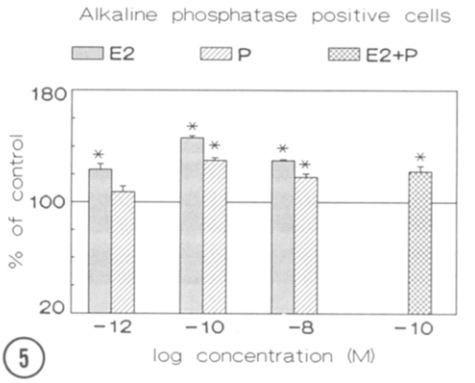


Fig. 4. Effects by 10^{-10}M E2 and 10^{-10}M progesterone on total biochemical alkaline phosphatase activity of the HOB cultures. Mean ± SEM relative to control values of 10-11 observations (2 experiments). * $P<0.01$ versus control values.

Fig. 5. Effects of E2 and progesterone on the number of cells showing AP activity as visualized by cytochemical staining of the HOB cultures. Mean ± SEM relative to control values of 10-12 cultures (2 experiments). * $P<0.05$ versus controls.

DISCUSSION

In the present study the effects of the hormones were investigated using human bone-derived cells pooled from different patients in order to minimize inter-patient variation. Thus, we are able to show here for the first time a direct stimulation of the growth and differentiation of normal adult HOB by E2 and progesterone in short-term serum-free cultures. Our results also emphasize that E2 tended to be a more differentiation-inducer (selective stimulation of HOB phenotype, i.e. AP activity), whereas progesterone was shown to be a growth-enhancer, stimulating both AP-positive as AP-negative cells. In 2-days' serum-free cultures, where unlike progesterone, E2 effects on cell proliferation was nihil, the differentiation effects were maintained. Further work studying other osteoblastic parameters are necessary to elucidate the differential effects of the hormones in this human bone cell system. Cells derived from male patients were also capable of resonding to the female steroid hormones, be it in a lower magnitude than the female cells did. Sex-specific actions of sex steroid hormones on rat bones and osteoblastic cells have been reported before (9). Recently, androgen (dihydrotestosterone) effects on proliferation and differentiation have been described for human osteoblast-like cells (21). We have found significant effect of testosterone on HOB derived from both male and female human bones (unpublished results). Taken together these observations strengthen the notion that sex steroids may directly play an important role in the regulation of the bone mass. Experimental evidence has recently been obtained in rats that estrogen,

next to its action of inhibiting bone resorption, may be able to maintain bone volume through stimulation of bone formation (22).

Insulin-like growth factors (IGF-I/IGF-II) and transforming growth factor β (TGFβ) have been implicated to be important local modulators of bone cell function (23). Data from the literature have indicated that steroid hormones may act on osteoblastic cells by promoting the production of these autocrine/paracrine growth factors (4,8,24,25). Recently, it has been reported that E2 inhibited the interleukin-1 (Il-1) and tumor necrosis factor-α induced Il-6 production by stromal and osteoblastic cells *in vitro*, pointing to a possible link of a direct effect of estrogen on osteoblasts to bone resorbing osteoclasts (26). This would confirm the finding that osteoblastic cells are required for inhibition of bone resorption by estrogen (27,28). Future studies should be aimed at clarifying the precise mechanisms by which the sex steroid hormones affect osteoblast function in conjunction with local factors.

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