17β-ESTRADIOL STIMULATES MINERALIZED BONE NODULE FORMATION WHEN ADDED INTERMITTENTLY TO SaOS-2 CELLS

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SUMMARY

It is now well established that estrogen inhibits bone resorption. However, its effect on bone formation remains controversial. We studied the effect of 17β-estradiol (E_2) on mineralized bone nodule formation in long-term cultures of osteosarcoma SaOS-2 cells. We showed that SaOS-2 cells formed mineralized nodules which under electron microscopy revealed a bone structure with active osteoblasts, entrapped osteocytes, extracellular collagen fibrils and hydroxyapatite deposits, making this system a valid model to study bone formation *in vitro*. Intermittent addition of E_2 for 6 hours during a 48-hour cycle of changes of medium, starting from day 3, resulted in a dose-dependent stimulation of mineralized bone nodule number and area, as well as alkaline phosphatase activity. In conclusion, we report for the first time a stimulatory effect of E_2 on mineralized bone nodule formation in human osteoblasts in culture.

KEY WORDS

estrogen, mineralized bone nodule, human osteoblasts, alkaline phosphatase, differentiation, SaOS-2 cells

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INTRODUCTION

Estrogen deficiency in postmenopausal women is now known to be a major cause of osteoporosis, a disease characterized by low bone mass and deterioration of the microarchitecture of bone tissue with a consequent risk of fracture. It is thought that the major effect of estrogen in postmenopausal women is inhibition of resorption /1/ (reviewed in /2,3/), but effects on bone formation have also been reported in both *in vivo* /3,4/ and *in vitro* /6/ studies in lower species. Since it is well known that there are species differences in the action of hormones on osteoblasts, we have undertaken a study of the effect of E_2 on bone formation using a human osteoblast-like cell system.

MATERIALS AND METHODS

Cell culture

SaOS-2 cells (obtained from American Type Culture Collection, Rockville, MD) were initially cultured in HAM's F-12 containing 10% fetal calf serum, HEPES buffer, pH 7.35, antibiotic-antimycotic, 2 mM glutamine and 1.1 mM $CaCl_2$, as previously described /7/. This was further supplemented with 10 nM dexamethasone (Dex) to promote the differentiation of SaOS-2 cells, as we have shown in a previous study /7/, and 50 μg/ml ascorbic acid, shown in previous studies to be important for matrix synthesis /8-10/. β-Glycerophosphate (10 mM), a source of organic phosphate shown to be important for *in vitro* mineralization /11/, was added from day 8 to the end of the culture period. Vehicle or varying concentrations of E_2 were added at day 3 for the first 6 h; the medium was then replaced fresh without the hormone, and the cultures continued for 48 h. The intermittent addition of vehicle or E_2 was repeated until day 15-17.

Determination of mineralized bone nodule formation

The cells were fixed overnight with neutral buffered formalin and stained *in situ* with von Kossa reagent. The mineralized nodule numbers and areas were quantified by LECO image analyser.

Alkaline phosphatase (ALP) activity assay

The cells were washed twice with 50 mM TRIS, pH 7.3, harvested by scraping, and ALP activity determined according to the method of Lowry /12/ using cell sonicates as previously described /7/. The protein content of the cell sonicate was determined using the commercial Biorad protein dye reagent.

Light microscopy, electron microscopy and electron microprobe analyses

von Kossa stained cells were examined and photographed with a camera fitted onto a Zeiss phase contrast microscope. Cells cultured for 15 days in cyclopore membrane (VWR) wells were prefixed overnight in 2.5% glutaraldehyde in Sorensen's phosphate buffer, pH 7.4, then processed according to standard protocols and analyzed with a Philips 410LS transmission electron microscope (TEM). Electron diffraction was performed on the same section on a Philips EM430 TEM.

RESULTS

An examination of the cultures of SaOS-2 cells under phase contrast light microscope at various periods of time in the presence of Dex, ascorbic acid and β-glycerophosphate showed that the cells started multilayering on day 6. By day 8, nodules comprised of clusters or clumps of polygonal cells with a three-dimensional structure were seen (data not shown). On day 17, the nodules were visible after von Kossa staining, as illustrated in the photograph of a culture dish in Figure 1A. A photomicrograph of stained nodules on day 17 revealing mineralization is shown in Figure 1B. An electron-micrograph of an ultrathin section of a representative nodule is shown in Figure 1C, demonstrating mineral deposits and banded collagen fibrils in the extracellular spaces. The mineral deposits had the characteristic crystalline structure of hydroxyapatite as demonstrated by the electron diffraction pattern in Figure 1D.

Mineralized bone nodule formation and the effects of E_2 were followed over time of culture of SaOS-2 cells. At each time point, the cells were fixed, stained with von Kossa reagent, and the nodule

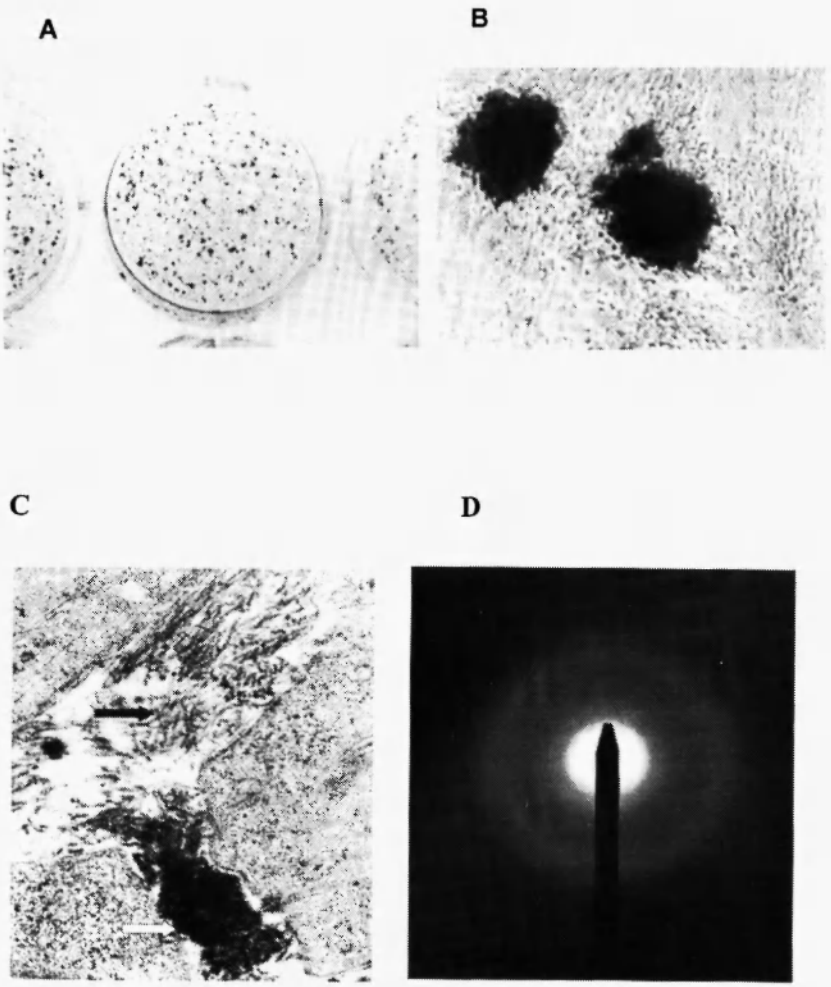

Fig. 1: **A.** Photograph of a culture dish of SaOS-2 cells demonstrates discrete mineralized nodules. **B.** Photomicrograph of mineralized nodules taken through a phase contrast microscope (x200). **C.** Electronmicrograph of an ultrathin section of a representative bone nodule illustrating banded collagen (black arrow) and mineral deposit (white arrow) (x21,000). **D.** Electron diffraction pattern demonstrating the characteristic crystalline structure of hydroxyapatite of the mineral deposit.

number and area quantified by image analyzer. The mineralized nodules could be visualized on day 10, the earliest time point examined, or at 2 days after the addition of β-glycerophosphate. The nodule number (Fig. 2A) and area (Fig. 2B) increased exponentially with time of culture up to 17 days, the last time point studied. Intermittent addition of 10 nM E_2 on day 3 and subsequently with a 48-hour cycle resulted in statistically significant stimulation of bone nodule number (Fig. 2A) (two-way ANOVA: for E_2 effect: n = 6, F = 1.7, p <0.0001, and for time-course effect: n = 6, F = 240.8, p <0.001) and nodule area (Fig. 2B) (two-way ANOVA: for E_2 effect: n = 6, F = 53.93, p <0.0001, and for time-course effect: n = 6, F = 117.5, p <0.0001) when compared with vehicle alone, in a time-dependent manner from days 13 to 17.

Figure 3 shows the dose-dependent effects of E_2 on the mineralized bone nodule formation in SaOS-2 cells at day 17 of culture. The graph illustrates that E_2 dose-dependently stimulated the mineralized nodule number (Fig. 3A) (one-way ANOVA: n = 6, F = 6.88, p <0.005) and nodule area (Fig. 3B) (one-way ANOVA: n = 6, F = 5.73, p<0.01) at 10 nM to 1 μM E_2.

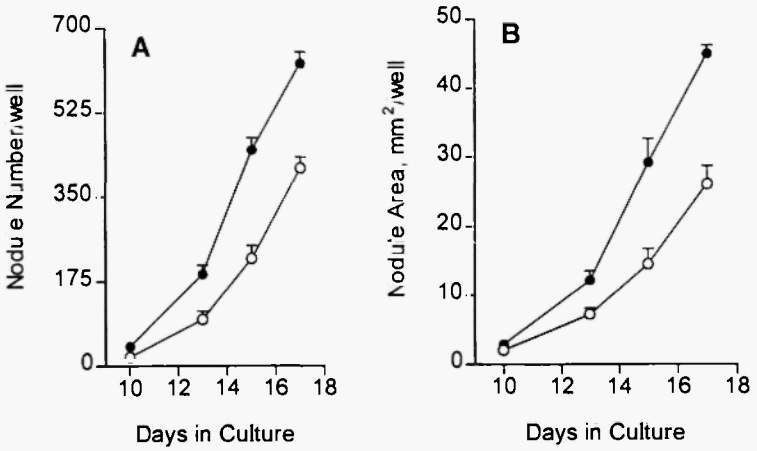

Fig. 2: Time course of the effect of E_2 on mineralized bone nodule number (**A**) and area (**B**). Vehicle (○) or 10 nM E_2 (●) was pulsed intermittently for 6 h every 48 h as described under Methods. At each time point, the cells were fixed and stained with von Kossa reagent, and the mineralized nodules quantified by image analyzer.

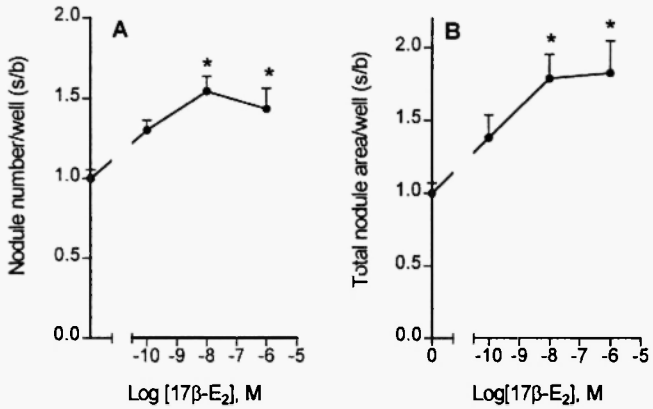

Fig. 3: Dose-dependent effects of E_2 on mineralized bone nodule number (**A**) and area (**B**). SaOS-2 cells were treated with intermittent addition of increasing concentrations of E_2 as described under Methods. * $p < 0.01$.

The development of ALP activity over time in culture and the effect of intermittent addition of E_2 are represented in Figure 4A. ALP activity was maximal on day 13, and slightly decreased by day 17. Intermittent addition of E_2 from day 3 showed a stimulatory effect on ALP activity over the time periods tested from day 3 to day 17 (Fig. 4A) (two-way ANOVA: for E_2 effect: $n = 6$, $F = 29.26$, $p < 0.0001$, and for time course effect: $n = 6$, $F = 190.6$, $p < 0.001$). A dose-dependent stimulation of ALP activity by intermittently added E_2 was observed from 10 nM to 1μM of E_2 (Fig. 4B) (one-way ANOVA: $n = 6$, $F = 18.08$, $p < 0.0001$). This stimulation correlated with the stimulation of bone nodule formation observed and illustrated in Figure 3A and B. In parallel experiments, 17α-estradiol was found to be ineffective (data not shown).

DISCUSSION

In this study we have shown for the first time that E_2 has a stimulatory effect on mineralized bone nodule formation in human osteosarcoma SaOS-2 cells which provides some evidence for a direct effect of E_2 on osteoblasts as a probable cause of the bone formation

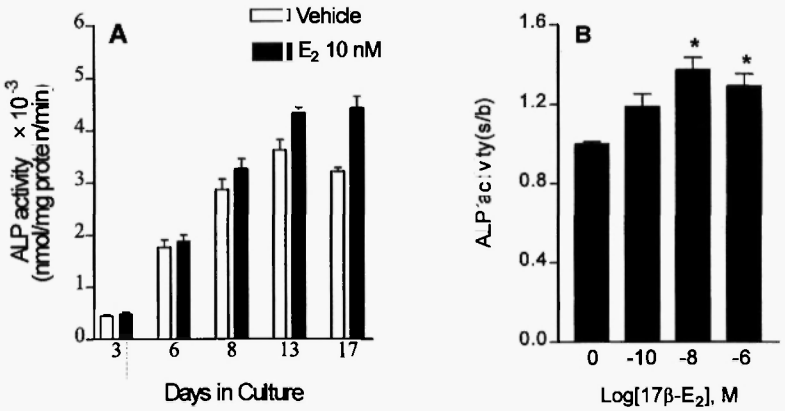

Fig. 4: Time course (**A**) and dose-dependent effects (**B**) of E_2 on ALP activity in SaOS-2 cells treated as described in Figs. 2 and 3 and in the text under Methods. * $p < 0.01$.

observed *in vivo* /3-5/. Our data also demonstrate that the long-term culture of SaOS-2 cells in the presence of Dex, β-glycerophosphate and ascorbic acid is a valid model to study bone formation *in vitro*.

SaOS-2 cells have been shown previously to deposit mineralized matrix in long-term culture /13/ and to spontaneously release matrix-vesicle-like structures with the capacity to mineralize /14/. However, although the matrix deposited has been extensively characterized /13/, discrete mineralized nodules capable of being quantified, and the regulation of mineralized nodule formation by estrogen, have not been reported. In this study, we used the system of long-term culture of SaOS-2 cells to study the effects of E_2 on bone formation *in vitro* by quantifying its effects on bone nodule number and area. We reported previously that continuous exposure of SaOS-2 cells to E_2 caused a small inhibition of both mineralized nodule number and area /15/. However, as we have shown in this study, intermittent exposure of the cells to E_2 resulted in stimulation. The stimulatory effect of intermittent addition of E_2 on bone nodule number could be a result of its effect on the differentiation of osteoprogenitor cells, as demonstrated by the parallel effect on ALP activity, a marker of osteoblast

differentiation. Bellows and Aubin /16/ showed that each nodule formed in rat calvarial osteoblast culture is derived from a single osteoprogenitor cell that proliferated and differentiated into mature bone-forming osteoblasts. Although SaOS-2 cells are a clonal cell line, they appear to have the potential and capacity to differentiate into various stages in culture /17,18/. Since E_2 was added on day 3, when osteoprogenitor number is still increasing (Fig. 2A), it can be concluded that the effect of E_2 is to promote further increase in the pool of osteoprogenitors and/or further differentiation of the cells into bone-forming mature osteoblasts. Our study cannot differentiate between these two mechanisms.

The reason for the difference in response to different treatments (continuous vs intermittent) with E_2 is not clear at the present time. However, such differences were also observed between continuous and intermittent treatment of osteoblasts with parathyroid hormone (PTH), albeit in cells from lower species /15/. It was shown in that study that PTH may be mediated by the signalling pathway of either protein kinase C (PKC) or protein kinase A (PKA), depending on the mode of addition /19/. Such a possibility in the case of E_2 action needs to be explored.

We have shown in earlier studies that culturing SaOS-2 cells in the presence of Dex caused an increase in immunohistochemically localized estrogen receptor (ER)-α /20/. In this study (data not shown), we found that E_2 did not have any influence on the lack of mineralized nodule formation when SaOS-2 cells were cultured in the absence of Dex. These data could imply that the effect of E_2 on bone formation we observed in this study is mediated via ER. The participation of ER-β may also be possible in view of the report that the expression of ER-β mRNA increased during differentiation and particularly at the mineralization stage of human osteoblast SV-HFO cells /21/. It is also possible that both ER-α and ER-β are involved in the stimulatory effect of E_2 seen in this study via heterodimerization, as previously suggested /22/.

In conclusion, we have shown that estrogen has a direct effect on mineralized bone nodule formation by osteoblasts. Estrogen is one of the most common drugs for the treatment of osteoporosis, but knowledge about its mechanism of action is still far from clear. The currently available evidence indicates that the major effect of estrogen is inhibition of bone resorption /1/ (reviewed in /2,3/). However, our

finding that E_2 has anabolic properties in human osteoblasts *in vitro* is in keeping with previous reports of the stimulatory effect of estrogen on bone formation seen in animal studies /4-6/. We hypothesize that part of the beneficial clinical skeletal effects of E_2 in bone may be the stimulation of early pathways in bone formation.

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