Journal of Cellular Biochemistry 73:500–507 (1999)

Comparative Effects of Estrogen and Antiestrogens on Differentiation of Osteoblasts in Mouse Bone Marrow Culture

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Abstract Estrogens as well as some antiestrogens have been shown to prevent bone loss in postmenopausal women. These compounds seem to inhibit bone resorption, but their anabolic effects have been less explored. In this study, bone marrow cultures were used to compare the effect of 17β-estradiol (E2), and two triphenylethylene derivatives, tamoxifen (TAM), and FC1271a, and a benzothiophene derivative raloxifene (RAL) on differentiation of osteoblasts. All enhanced osteoblastic differentiation of 21-day cultures as indicated by increased mineralization and bone nodule formation. All, except RAL, stimulated cell proliferation during the first 6 days of the culture. However, in the presence of RAL the content of total protein was increased in 13-day cultures. SDS-PAGE and autoradiography of [^{14}C]-proline labeled proteins revealed elevated level of the newly synthesized collagen type I. The pure antiestrogen ICI 182,780 abolished the increase of the specific activity of alkaline phosphatase by E2, TAM, and FC1271a but not the effect of RAL on protein synthesis. Our results show that E2 as well as TAM, FC1271a, and RAL stimulate bone formation in vitro but the mechanism of the anabolic action of RAL in bone clearly differs from that of E2, TAM, and FC1271a. J. Cell. Biochem. 73:500–507, 1999.

Key words: osteoblast; estrogen; raloxifene; tamoxifen; FC1271a

Estrogen decrease in postmenopausal women results in an increase of bone turnover, with bone resorption exceeding bone formation. Eventually this leads to a general loss of bone mass [Riggs, 1991]. Estrogen has been shown to be effective in preventing postmenopausal bone loss [Riggs and Melton III, 1992], but its use is limited by the concern of increasing risk of development of endometrial and breast cancer. Meanwhile, several synthetic antiestrogens, which present both estrogen agonist and antagonist properties, have been found to have beneficial effect on bone. These antiestrogens include triphenylethylene derivatives (e.g., clomiphene, tamoxifen, droloxifene, toremifene) and benzothiophene derivatives (e.g., raloxifene). Several studies have demonstrated that tamoxifen maintains bone mass in ovariectomized rats [Jordan et al., 1987; Turner et al., 1987, 1988]. Also, raloxifene has been reported to prevent bone loss, while not showing any significant stimulatory effect on uterus [Larry et al., 1994]. FC1271a is a novel triphenylethylene derivative with a tissue-specific profile in vivo. FC1271a specifically binds to the estrogen receptor with the affinity closely similar to that of TAM [Härkönen et al., 1996].

The presence of estrogen receptors α and β in osteoblasts [Eriksen et al., 1988; Arts et al., 1997] suggests that estrogen has a direct role in the regulation of osteoblast function. However, the in vitro studies carried out to demonstrate the effect of estrogen on osteoblasts and bone formation have yielded conflicting results. In the cells of the osteoblastic lineage from a variety of sources, estrogen has been reported to stimulate, to inhibit, or not to affect osteoblast proliferation and/or differentiation [Ernst et al., 1988; Watts et al., 1989; Keeting et al., 1991; Scheven et al., 1992]. Although many of the in vivo studies have demonstrated that the predominant effect of estrogen on bone remodeling is to decrease bone resorption [Riggs et al., 1972; Parfitt, 1979], some have suggested that

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Received 2 November 1998; Accepted 6 January 1999

estrogen may also increase bone formation directly [Turner, 1991; Chow et al., 1992a]. By using an in vitro model we have demonstrated recently that estrogen stimulates sequential differentiation of osteoblasts and increases deposition and mineralization of matrix in mouse bone marrow cultures [Qu et al., 1998]. Studies with a human fetal osteoblastic cell line also suggest that estrogen can directly regulate osteoblast differentiation [Robinson et al., 1997]. Recent report of Takeuchi et al. [1995] indicated that tamoxifen enhances the mineralization of matrix deposited by human osteosarcoma cells.

The recent discovery of an additional estrogen receptor subtype (ERβ) in various tissues can, at least partially, explain the pleiotrophic effects of estrogen. A marked expression of ERβ has been shown in prostate and ovary [Kuiper et al., 1996]. Recently, expression of ERβ has been observed in osteoblasts and other bone cells, and the level of expression was higher than that of ERα [Arts et al., 1997; Onoe et al., 1997]. It has been reported that most estrogen and anti-estrogen compounds bind to both known subtypes of ER (ERα and ERβ) with almost identical affinity (Mosselman et al., 1996; Kuiper et al., 1997). Although ERβ transactivates promoters containing estrogen responsive elements (ERE) in an estradiol-dependent manner, the regulation of the transcriptional activity through ERβ appears to be distinct from that of ERα [Tremblay et al., 1997].

Besides the ER-mediated regulation of gene transcription through the estrogen response element (ERE), it is possible that RAL and TAM can modulate bone cell activities through pathway(s) independent of ERE [Takeuchi et al., 1995; Yang et al., 1996a]. The ligand-activated ERα and ERβ can also signal via AP1 site and in certain cellular contexts can in opposite ways [Paech et al., 1997]. In transfected HeLa cells, E2 bound to ERα activated transcription of the reporter luciferase gene whereas ERβ inhibited it [Paech et al., 1997]. Moreover, the antiestrogens like tamoxifen and raloxifene were also demonstrated to be potent transcriptional activators with ERβ at an AP1 site [Paech et al., 1997]. In addition, the activation of human transforming growth factor-β3 gene in intact bone and cultured bone cells by RAL has been shown to occur through the mediation of raloxifene response element (RRE), and this regulation does not require the DNA binding domain of the ER [Yang et al., 1996a]. Also, TAM has been reported not to activate ERE-mediated transcription in osteoblasts, although it stimulates mineralization by human osteoblast-like osteosarcoma cells as an estrogen agonist [Takeuchi et al., 1995].

In this study, we have compared the effects of tamoxifen, raloxifene, and a new triphenylethylene derivative FC1271a to that of 17β-estradiol during the differentiation of osteoblasts derived from mouse bone marrow stromal cells.

MATERIALS AND METHODS

Cell Cultures

All cultures were carried out in phenol red-free α-MEM (Gibco, New York, NY) supplemented with 15% fetal calf serum (BIOCLEAR, Bioclear UK Ltd., Wilts, UK), 10 nM dexamethasone (Sigma Chemical Co., St. Louis, MO), ascorbic acid (50 μg/ml), 10 mM sodium β-glycerophosphate and antibiotics in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. FC1271a, raloxifene (RAL), and tamoxifen (TAM) were kindly provided by Dr. Marja Södervall (Orion Corporation, Oulu, Finland). The pure antiestrogen ICI 182,780 was a gift from Dr. AE Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK). 17β-estradiol (Sigma Chemical Co.) and the above compounds were dissolved in ethanol as a 10 mM stock solution. The final concentration of ethanol in culture medium was 0.01% or less. Control cultures received ethanol to the same final concentration.

Bone marrow cells were obtained from the femurs of 10-week-old female NMRI mice bred in the Animal Center of University of Oulu. Animals were killed by cervical dislocation. Both femora were removed and the soft tissues were detached aseptically. Metaphyses from both ends were resected and bone marrow cells were collected by flushing the diaphysis with culture medium. A suspension of bone marrow cells was obtained by repeated aspiration of the cell preparation through a 22 gauge needle and nucleated cells were counted with a hemocytometer. Cells were plated in 260 ml Tissue Culture Flasks (Nunc, Roskilde, Denmark) at a density of 10^6 cell/cm^2 and cultured for 6 days by replacing the medium every 3 days (phase I). On day 6, subcultures were prepared and the cultures were continued for various time periods (phase II). Cells were washed with warm PBS and adherent cells were detached by using trypsin-

EDTA (Sigma-Aldrich Co. Ltd., Irvine, UK). Trypsinized cells were passed through a syringe with a 22 gauge needle to make a single-cell suspension, counted and plated in 24-well or six-well plates at a density of 5×10^3 cells/cm^2. The next day E2, or TAM, or FC1271a, or RAL treatment was started. For studying the cell proliferation bone marrow cells were plated directly in 6-well plates at a density of 5×10^3 cells/cm^2 and cultures were continued without subculturing for 6 days.

Cell Proliferation Assay

To assess the effects of E2, TAM, FC1271a, and RAL on cell proliferation during phase I of the cultures, bone marrow cells were dispersed into 6-well culture plates (Nunc, Roskilde, Denmark) at a density of 10^4 cell/cm^2. The above compounds were added at different concentrations ranging from 0.01–10 nM. On day 6, the cells were trypsinized and cell numbers counted using a hemocytometer.

Assay of Cellular Alkaline Phosphatase (ALP) Activity

Cellular ALP activity was assayed by the method of Lowry et al. [1954]. The cell layers were washed twice with PBS, and extracted into 200 µl 0.1% Triton X-100 buffer (pH 7.6). They were then frozen and thawed three times, and sonicated in an ice bath for 30 sec using an ultrasonic disruptor. Enzyme activity was determined colorimetrically using *p*-nitrophenylphosphate as substrate at pH 9.7 and reading the optical density at 405 nm. In parallel, the protein contents of the wells were determined by BIO-RAD protein assay (Bio-Rad Laborotaries, Richmond, CA) and ALP activity was expressed as units/mg protein.

[^{14}C]-Proline Incorporation Assay for Matrix Protein

Protein synthesis in the subcultures (phase II) was studied by using 96-well plates at a density of 10^4 cell/well. On day 13 the cells were pulsed for 12 h with 1 µCi/ml [^{14}C]-proline in 200 µl proline-free medium. Following a 12-h pulse, the relative number of cells in each well was determined using a colorimetric method with a CellTiter 96 Kit (Promega, Madison, WI). Absorbance was read at 490 nm and the relative cell numbers (relative absorbances) calculated on the basis of the standard curve. The medium was then removed and the cell layer was washed with PBS. The matrix proteins were extracted with 1% SDS (Bio-Rad Laboratories) and proteins were precipitated with an equal amount of 20% TCA (Baker Analyzed® Reagent, Deventer, Holland). After centrifugation, the protein pellet was dissolved in 100 µl of 1% SDS. Radioactivity was measured from a 50 µl aliquot of the solution by a Liquid Scintillation Counter (LKB-Wallac, Finland).

To characterize the proportion of newly-synthesized major proteins, subcultures in 24-well plates were pulsed for 24 h at day 13 with 1 µCi/ml [^{14}C]-proline in a proline-free medium. The matrix proteins were extracted with 200 µl of 1% SDS and the radioactivity was determined by a Liquid Scintillation Counter. Twenty µg of protein from each sample was loaded on 9.5% SDS-PAGE. The size of proteins was estimated by using a commercial kit of standards (Sigma Chemical Co.). The gel was immersed with 20% 2,5-diphenyl-oxazole (Sigma), dried, and exposed to an X-ray film.

Calcium Determination and Detection of Bone Nodules

Calcium content in the cultures was determined as described elsewhere [Gitelman, 1967]. The cell cultures were washed three times with Ca^{2+}- and Mg^{2+}-free PBS and incubated overnight at room temperature in 0.6 N HCl. Extracts of 50 µl were complexed with 1 ml o-cresol-phthalein-complexon (Test Combination Calcium, Boehringer-Mannheim, Mannheim, Germany). The colorimetric reaction was read at 570 nm in a spectrophotometer. For detection of bone nodules, cultures were fixed with 40% ethanol for 20 min. Bone nodules were detected with von Kossa [von Kossa, 1901] staining for calcium. After staining, the proportional area of positive staining was analyzed by digital image analysis (M2 image analyzer, Imaging Research Inc., Brock University, Ontario, Canada).

Statistical Analysis

Data were expressed as mean ± SEM. Statistical significance was determined using an one-way analysis of variance (ANOVA) and the unpaired Student t-test to compare means between groups, with a P value of less than 0.05 being considered significant.

RESULTS

Effect of E2 and Antiestrogens on Bone Marrow Cell Proliferation

We have shown earlier that using this model E2 stimulates cell proliferation during the phase I with concentrations ranging from 0.01 nM to 10 nM with a peak at 0.1 nM [Qu et al., 1998]. Figure1 shows that TAM increased cell proliferation with the concentrations ranging from 0.1 to 10 nM. The maximal effect was achieved at a concentration of 1 nM TAM. A novel triphenylethylene derivative, FC1271a, also increased cell proliferation during the phase I of the cultures with a profile similar to that of E2 (Fig. 1). When compared to controls, maximal stimulation of cellular proliferation achieved with E2, FC1217a, and TAM was 81%, 48%, and 42%, respectively. Opposite to all other compounds studied RAL had no effect on cell proliferation at any concentrations (1 nM–1 μM) tested (Fig. 1).

Effect of E2 and Antiestrogens on Differentiation of Osteoblasts

ALP expression is a typical marker of osteoblast differentiation and was followed here by measuring total and specific activity of the enzyme. E2 was found to increase the specific activity of ALP with a maximal effect at 0.1 nM as also reported earlier [Qu et al., 1998] (Fig. 2a). With E2, the increase in the specific activity was due to the relative increase of the expression of ALP. TAM and FC1271a also resulted in significant increase in specific activity of ALP, showing maximal effect with the concentrations of 1 nM and 0.1 nM, respectively (Fig. 2b,c). The stimulatory effect of E2, TAM, and FC1271a on ALP activity could be opposed by a pure antiestrogen ICI 182,780 (Fig. 2). In RAL treated cultures we first observed a dose-dependent increase of total ALP activity (Fig. 3a) but this was not, however, reflected as an increase in the specific activity (Fig. 3b), as with other compounds. In contrast it paralleled with a marked increase of total protein content of cultures (Fig. 3c). The increase of total pro-

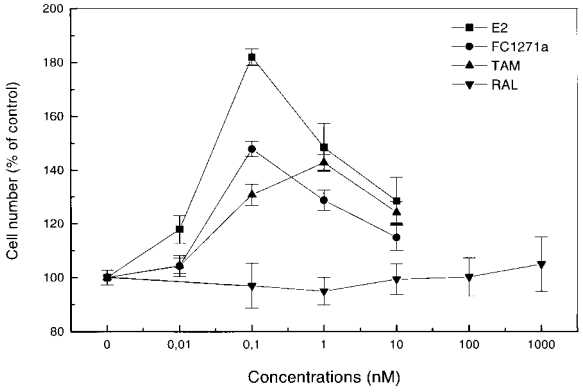

Fig. 1. Effect of E2, TAM, FC1271a, and RAL on cell proliferation in mouse bone marrow derived cultures at day 6. Means ± SEM (n = 6) are expressed in relation to values in control cultures.

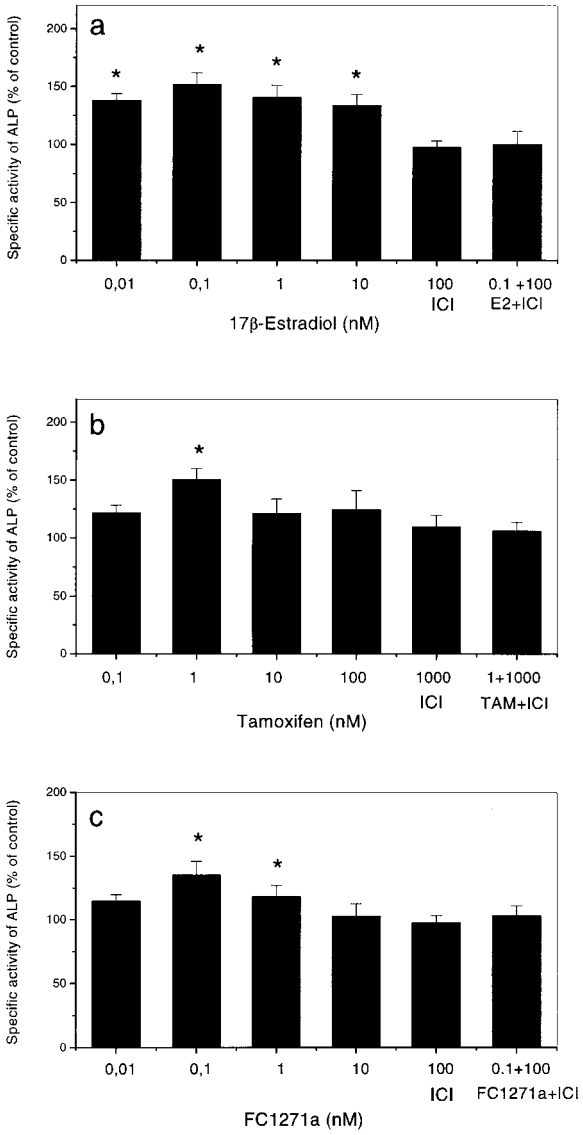

Fig. 2. Effect of E2 (**a**), TAM (**b**), and FC1271a (**c**) on the specific activity of ALP in mouse bone marrow derived cultures as measured on culture day 15. The columns show the means ± SEM from three different experiment each containing four parallel wells. In all cases addition of ICI 182,780 was able to block the stimulation. $*P < 0.05$ vs. controls.

tein content was not observed with either E2 or with TAM or FC1271a. RAL-induced increase in total protein content of cultures was decreased by addition of ICI 182,780 to the incubation medium with RAL but the different was not statistically significant (Fig. 4a). ICI 182,780 alone did not cause any change. In order to test this observation further, we measured [^{14}C]-proline incorporation into protein in cultures grown in the presence of different concentrations of RAL. As shown in Figure 4b, RAL increased the incorporation of [^{14}C]-proline into protein in a concentration dependent manner. This stimulatory effect on proline incorporation could not be found in E2-, FC1271a-, or TAM-treated cultures (data not shown).

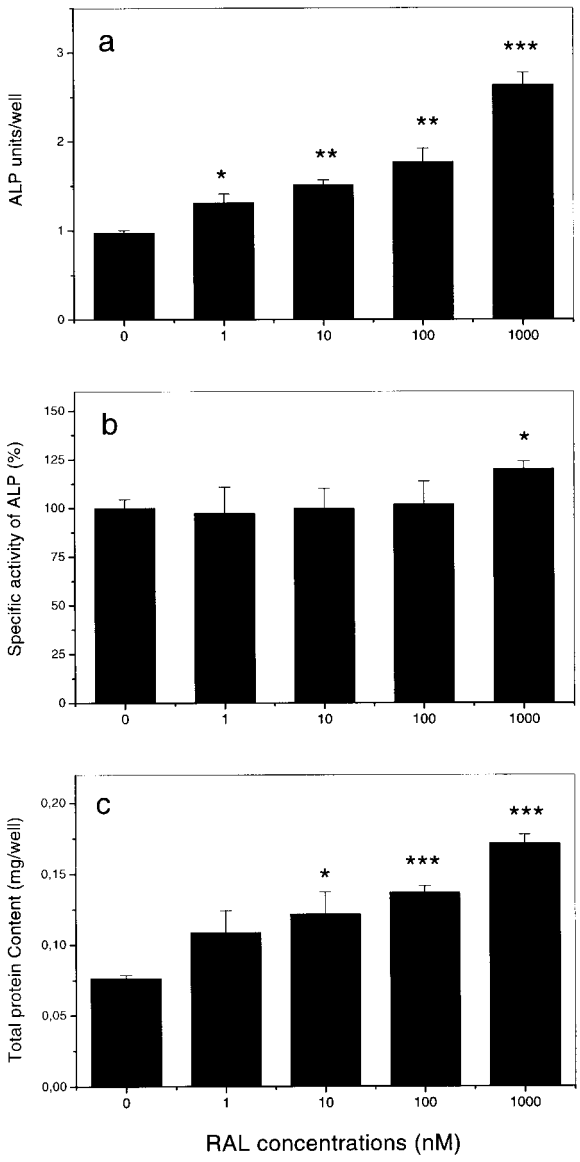

Fig. 3. Effect of different concentrations of RAL on ALP activity in mouse bone marrow derived cultures measured on culture day 15. Marked increase of enzyme activity (**a**) was not due to the increase of specific activity of ALP (**b**), but due to the increase in total protein content of cultures (**c**). The columns show the means ± SEM from three separate experiments each containing four parallel wells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. controls.

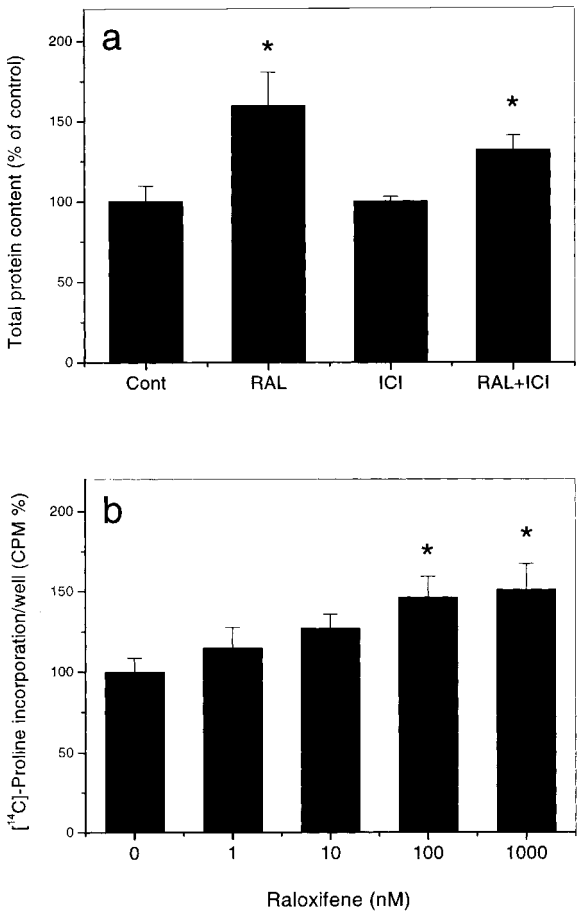

Fig. 4. **a**: Effect of ICI 182,780 (1 μM) on RAL (10 nM) induced increase of total protein content in mouse bone marrow derived cultures at day 13. **b**: Linear increase in [^{14}C]-proline incorporation at the same time point. The columns show the means ± SEM from three experiments each containing four parallel wells. *$P < 0.05$ vs. controls.

We next studied the relative incorporation of [^{14}C]-proline into newly synthesized proteins by using SDS-PAGE and autoradiography of the gels (Fig. 5). Coomassie blue staining of the reduced SDS-PAGE revealed the bands of α1 and α2 chains of type I collagen with a ratio of about 2:1 (Fig. 5a). RAL treatment enhanced the relative staining intensity of collagen α1 and α2 bands (Fig. 5a). Autoradiography further supported the increased incorporation of [^{14}C]-proline into several protein bands includ-

ing type I collagen α1 and α2 chains in the cultures grown in the presence of RAL (Fig. 5b).

Effect of E2 and Antiestrogens on Matrix Mineralization

To analyze the final differentiation status of cultures at day 21, we stained the cultures with von Kossa method to visualize calcium deposition. Positively stained areas were clearly increased by all the compounds studied when compared to the control (Fig. 6a). We also measured the proportional area of von Kossa positive staining (Fig. 6b) and calcium content of the cultures (Fig. 6c). All these measurements indicated significantly increased calcium apposition in the presence of E2, TAM, FC1271a, or RAL. However, we could not find clear differences in terms of bone nodule formation or calcium content between TAM, FC1271a, and RAL in the cultures.

DISCUSSION

E2 has been shown to have a stimulatory effect on bone formation and an inhibitory effect on bone resorption in vivo [Takano-Yamamato et al., 1990; Chow et al., 1992b,c; Harris et at., 1996]. Results of the in vitro experiments with osteoblasts have been controversial [Ernst et al., 1988; Watts et al., 1989; Keeting et al., 1991; Scheven et al., 1992]. We have previously reported [Qu et al., 1998] the expression of ERα in mouse bone marrow cultures and shown that E2 stimulates osteoblast differentiation and bone nodule formation through an ER-mediated manner. In this study we demonstrated that E2, TAM, and FC1271a stimulate bone marrow cell proliferation, specific activity of alkaline phosphatase and matrix mineralization in our in vitro model for bone formation. In contrast, RAL did not stimulate bone marrow cell proliferation but markedly increased matrix protein synthesis at the early stages of the culture. Although bone nodule formation and calcium accumulation eventually increased in

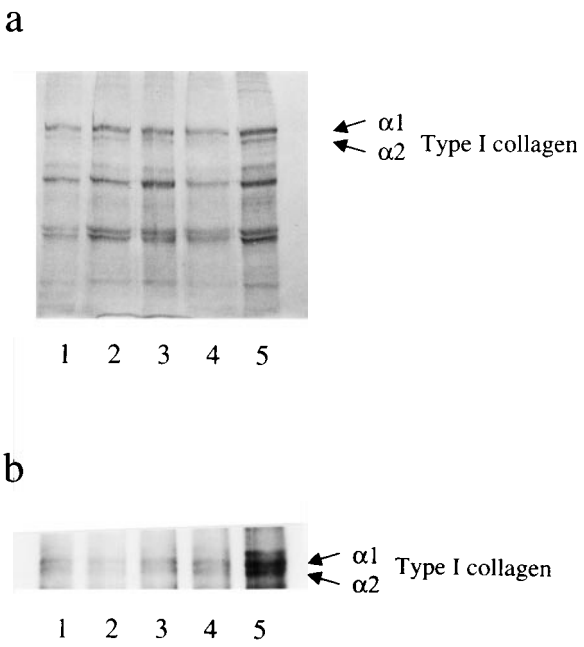

Fig. 5. **a**: SDS-PAGE revealed α1 and α2 chains of type I collagen in extractions of matrix protein. (**1**) Control, (**2**) E2 (0.1 nM), (**3**) FC1271a (0.1 nM), (**4**) TAM (1 nM), and (**5**) RAL (10 nM). **b**: Autoradiography showed the increased incorporation of [^{14}C]-proline into type I collagen α1 and α2 chains in the cultures grown in the presence of RAL. Lanes are labeled same as above.

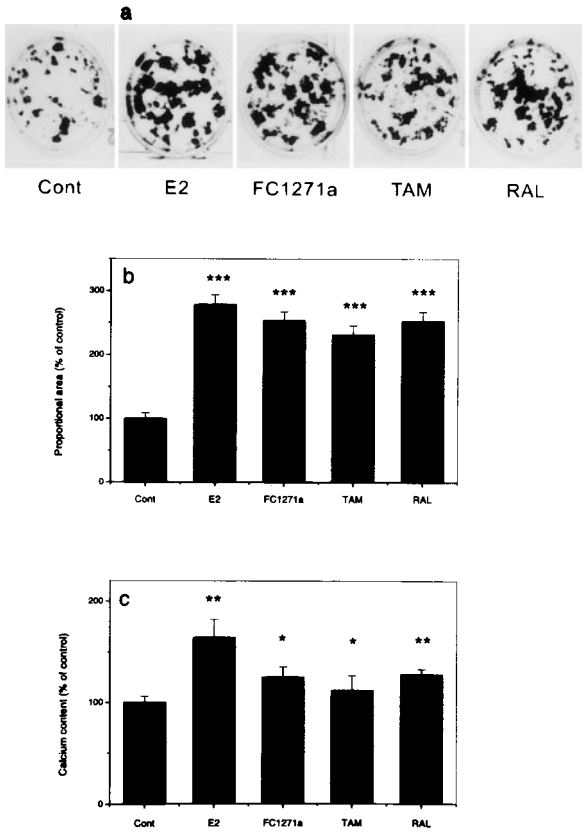

Fig. 6. Comparison of effects of E2, FC1271a, TAM, and RAL at doses of 0.1 nM, 0.1 nM, 1 nM, and 10 nM respectively, on matrix mineralization at day 21 in mouse bone marrow derived cultures. **a**: Bone nodule formation visualized by von Kossa staining. **b**: Proportional area of positive histological staining with von Kossa method. **c**: Total calcium content of cultures. The results are means ± SEM from three separate experiments each containing four parallel wells. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. controls.

the presence of all compounds studied, our results strongly suggest that the mechanism of action of RAL in bone marrow cultures differs from that of estrogen and other antiestrogens. This is in agreement with recent results of Nuttall et al. [1998], reporting that RAL showed no agonist activity through ERE in osteosarcoma cells.

Histomorphometric studies have shown that E2 and TAM treatment can similarly protect bone against OVX-induced bone loss [Turner et al., 1988; Larry et al., 1994]. However, recent in vitro experiments have also suggested that TAM can stimulate mineralization in human osteoblast-like osteosarcoma cell cultures [Takeuchi et al., 1995; Fournier et al., 1996]. In our study, TAM as well as a novel triphenylethylene derivative FC1271a enhanced cell proliferation and differentiation of bone marrow derived osteoblasts, suggesting that these synthetic analogs exert estrogen agonist activities on bone marrow cells.

The effect of RAL on bone in OVX rats is very similar to that of TAM. RAL antagonizes the changes induced by OVX in cortical and cancellous bone volume [Evans et al., 1994], bone density [Sato et al., 1994], bone mass [Jordan et al., 1987], and bone strength [Turner et al., 1994]. RAL has been reported to be able to restore the estrogen withdrawal-induced reduction of TGFβ3 mRNA levels in rat femur and to activate TGFβ3 promoter as a full agonist [Yang et al., 1996b]. More recently, it was found that TGFβ3 activation by RAL was not mediated by ERE but through a novel sequence, RRE, with which ER probably interacts by intervening proteins [Yang et al., 1996a]. In our experiments effects of RAL clearly differed from those of other antiestrogens since RAL did not stimulate cell proliferation during the early culture period, as E2 and other tested compounds did. However, RAL appeared to stimulate protein synthesis including type I collagen synthesis of differentiated osteoblasts. Furthermore, the stimulation by RAL could not be significantly opposed by a pure antiestrogen ICI 182,780. Finally, although RAL preferentially stimulated matrix protein synthesis in a unique manner, the end result of cultures at day 21 was not different from that of E2 and triphenylethylene derivatives. All these observations suggest that the mechanism of RAL action in bone differs from that of other antiestrogens studied.

It has been suggested that RAL is more potent than E2 in the regulation of TGFβ3 gene expression [Yang et al., 1996b]. In the TGFβ3 promoter area, the preferred ligand was RAL, whereas E2 activated the TGFβ3 promoter as a partial agonist/antagonist [Yang et al., 1996b]. In addition, the metabolites of E2 were found to function as effective agonists at TGFβ3 promoter [Yang et al., 1996b]. Thus, it is possible that RAL mimics the endogenous ligands by regulating production of matrix proteins in osteoblast cultures. It is now generally accepted that the main effect of E2 in the prevention of bone loss is via the bone resorption [Riggs et al., 1972; Parfitt, 1979]. Our in vitro results with osteoblasts suggest that the bone sparing effect of RAL may be partly due to its direct effect on osteoblasts and bone formation. It is thus important to study in details the effects of RAL and other estrogen analogues separately on bone resorbing and bone forming cells. This may help to understand their mechanisms of action at the molecular level and finally their effects in vivo.

In conclusion, our present results support the earlier results [Qu et al., 1998] that estrogen can enhance bone formation in vitro. We further demonstrate that TAM, FC1271a, and RAL have estrogen-like effects on bone formation in bone marrow cell cultures but the mechanism of action of these compounds seem to differ from each other.

ACKNOWLEDGMENT

We would like to thank Mrs. Marja Välimäki for her expert technical assistance.

REFERENCES

Arts J, Kuiper GGJM, Janssen JMMF, Gustafsson J-Å, Löwik CWGM, Pols HAP, van Leeuwen JPTM. 1997. Differential expression of estrogen receptors α and β mRNA during differentiation of human osteoblast SV-HFO cells. Endocrinology 138:5067–5070.

Chow JWM, Lean JM, Chambers TJ. 1992a. 17β-Estradiol stimulates cancellous bone formation in female rats. Endocrinology 130:3025–3032.

Chow J, Tobias JH, Colston KW, Chambers TJ. 1992b. Estrogen maintains trabecular bone volume in rats not only by suppression of bone resorption but also by stimulation of bone formation. J Clin Invest 89:74–78.

Chow JWM, Lean JM, Chambers TJ. 1992c. 17β-estradiol stimulates cancellous bone formation in female rats. Endocrinology 130:3025–3032.

Eriksen EF, Colvard DS, Berg NG, Graham ML, Mann DG, Spelsberg TC, Riggs BL. 1988. Evidence of estrogen receptors in normal human osteoblast-like cells. Science 241: 84–86.

Ernst M, Schmid CH, Froesch ER. 1988. Enhanced osteoblast proliferation and collagen gene expression by estradiol. Proc Natl Acad Sci USA 85:2307–2310.

Evans GL, Bryant HU, Magee D, Sato M, Turner RT. 1994. The effects of raloxifene on tibia histomorphometry in ovariectomized rats. Endocrinology 134:2283–2288.

Fournier B, Häring S, Kaye AM, Sömjen D. 1996. Stimulation of creatine kinase specific activity in human osteoblast and endometrial cells by estrogens and antiestrogens and its modulation by calciotropic hormones. J Endocrinology 150:275–285.

Gitelman HJ. 1967. An improved automated procedure for the determination of calcium in biological specimens. Anal Biochem 18:521–531.

Harris SA, Tau KR, Turner RT, Spelsberg TC. 1996. Estrogen and progestins. In: Bilezikian JP, Raitz LG, Rodan GA, editors. Principles of bone biology. San Diego: The Academic Press. p 507–520.

Härkönen PL, Qu Q, Dahllund JC, Laine A, Hemminki K, Kangas L, Väänänen HK. 1996. FC1271a is a novel tissue-specific antiestrogen with bone-sparing effect. 10th International Congress of Endocrinology Vol I:P1.256.

Jordan VC, Phelps E, Lindgren JU. 1987. Effects of antiestrogens on bone in castrated and intact female rats. Breast Cancer Res Treat 10:31–35.

Keeting PE, Scott RE, Colvard DS, Han IK, Spelsberg TC, Riggs BL. 1991. Lack of a direct effect of estrogen on proliferation and differentiation of normal human osteoblast-like cells. J Bone Miner Res 6:297–304.

Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson J-Å. 1996. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93:5925–5930.

Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson J-Å. 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 138: 863–870.

Larry JB, Sato M, Rowley ER, Magee DE, Bekele A, Williams DC, Cullinan GJ, Bendele R, Kauffman RF, Bensch WR, Frolik CA, Termine JD, Bryant HU. 1994. Raloxifene (LY139481 HCl) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. J Clin Invest 93:63–69.

Lowry OH, Roberts NR, Wu M, Hixton WS, Crawford EJ. 1954. The quantitative histochemistry of brain. II. Enzyme measurements. J Biol Chem 207:19–37.

Mosselman S, Polman J, Dijkema R. 1996. ERβ: Identification and characterization of a novel human estrogen receptor. FEBS Lett 392:49–53.

Nuttall ME, Bradbeer JN, Stroup GB, Nadeau DP, Hoffman SJ, Zhao H, Rehm S, Gowen M. 1998. Idoxifene: A novel selective estrogen receptor modulator prevents bone loss and lowers cholesterol levels in ovariectomized rats and decreases uterine weight in intact rats. Endocrinology 139:5224–5234.

Onoe Y, Miyaura C, Ohta H, Nozawa S, Suda T. 1997. Expression of estrogen receptor β in rat bone. Endocrinology 138:4509–4512.

Paech K, Webb P, Kuiper GGJM, Nilsson S, Gustafsson J-Å, Kushner PJ, Scanlan TS. 1997. Defferential ligand activation of estrogen receptors ERα and ERβ at AP1 sites. Science 277:1508–1510.

Parfitt AM. 1979. Quantam concept of bone remodeling and turnover: Implications for the pathogenesis of osteoporosis. Calc Tissue Int 28:1–5.

Qu Q, Perälä-Heape M, Kapanen A, Dahllund J, Salo J, Väänänen HK, Härkönen P. 1998. Estrogen enhances differentiation of osteoblasts in mouse bone marrow culture. Bone 22:201–209.

Riggs BL, Jowsey J, Goldsmith RS, Kelly PJ, Hoffman DL, Arnaud CD. 1972. Short-and long-term effects of oestrogen and synthetic anabolic hormone in postmenopausal osteoporosis. J Clin Invest 51:1659–1663.

Riggs BL. 1991. Overview of osteoporosis. West J Med 154:63–77.

Riggs BL, Melton III LJ. 1992. The prevention and treatment of osteoporosis. N Engl J Med 327:620–627.

Robinson JA, Harris SA, Riggs BL, Spelsberg TC. 1997. Estrogen regulation of human osteoblastic cell proliferation and differentiation. Endocrinology 138:2919–2927.

Sato M, McClintock C, Kim J, Turner CH, Bryant HU, Magee D, Slemenda CW. 1994. Dual-energy x-ray absorptiometry of raloxifene effects on the lumbar vertebrae and femora of ovariectomized rats. J Bone Miner Res 9:715–724.

Scheven BAA, Damen CA, Hamilton NJ, Verhaar HJJ, Duursma SA. 1992. Stimulatory effects of estrogen and progesterone on proliferation and differentiation of normal human osteoblast-like cells in vitro. Biochem Biophys Res Commun 186:54–60.

Takano-Yamamoto T, Rodan GA. 1990. Direct effects of 17β-estradiol on trabecular bone in ovariectomized rats. Proc Natl Acad Sci USA 87:2172–2176.

Takeuchi M, Tokin M, Nagata K. 1995. Tamoxifen directly stimulates the mineralization of human osteoblast-like osteosarcoma cells through a pathway independent of estrogen response element. Biochem Biophys Res Commun 210:295–301.

Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F, Giguere V. 1997. Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. Mol Endocrinol 11:353–365.

Turner CH. 1991. Do estrogens increase bone formation? Bone 12:305–306.

Turner CH, Sato M, Bryant HU. 1994. Raloxifene preserves bone strength and bone mass in ovariectomized rats. Endocrinology 135:2001–2005.

Turner RT, Wakley GK, Hannon KS, Bell NH. 1987. Tamoxifen prevents the skeletal effects of ovarian deficiency in rats. J Bone Miner Res 2:449–456.

Turner RT, Wakley GK, Hannon KS, Bell NH. 1988. Tamoxifen inhibits osteoclast-mediated resorption of trabecular bone in ovarian hormone-deficient rats. Endocrinology 122:46–50.

Von Kossa J. 1901. Uber die im organismus kunztlich erzeugen Verkalkungen. Beitr Anat 29:163.

Watts CKW, Parker MG, King RJB. 1989. Stable transfection of the oestrogen receptor gene into a human osteosarcoma cell line. J Steroid Biochem 34:483–490.

Yang NN, Venugopalan M, Hardikar S, Glasebrook A. 1996a. Identification of an estrogen response element activated by metabolites of 17 beta-estradiol and raloxifene. Science 273:1222–1225.

Yang NN, Bryant HU, Hardikar S, Sato M, Galvin RJ, Glasebrook AL, Termine JD. 1996b. Estrogen and raloxifene stimulate transforming growth factor-beta 3 gene expression in rat bone: A potential mechanism for estrogen- or raloxifene-mediated bone maintenance. Endocrinology 137:2075–2084.