JOURNAL OF BONE AND MINERAL RESEARCH
Volume 16, Number 2, 2001

Localization of Estrogen Receptor β Protein Expression in Adult Human Bone

ISOBEL P. BRAIDMAN,[1] LINDA HAINEY,[1] GAURAV BATRA,[1] PETER L. SELBY,[1] PHILIPPA T.K. SAUNDERS,[2] and JUDITH A. HOYLAND[1]

ABSTRACT

Evidence suggests that the newly described estrogen receptor β (ER-β) may be important for estrogen (17β-estradiol) action on the skeleton, but its cellular localization in adult human bone requires clarification. We addressed this by using indirect immunoperoxidase with a novel affinity purified polyclonal antibody to human ER-β, raised to hinge domain (D) sequences from the human receptor. Bone was demineralized in 20% EDTA and all biopsy specimens were formalin-fixed and wax-embedded. Vigorous retrieval was essential for ER-β detection. In sections (5 μm) of benign prostate hyperplasia, used as positive control, clear nuclear immunoreactivity was seen in glandular epithelial cells, with a 1:500 dilution of ER-β40. For bone sections, optimal antibody dilutions were 1:100–1:250. We found that in normal bone (from graft operations), in fracture callus from both men and women (>25 years old), pagetic bone, osteophytes, and secondary hyperparathyroid bone, all from older patients, ER-β was expressed clearly in osteoclast nuclei, with little cytoplasmic immunoreactivity. Nuclear immunoreactivity was still prominent in osteoclasts, with antibody diluted 1:500, although it faded in other cells. Osteoblasts, in areas of active bone formation or bone remodeling, also expressed ER-β, as did some osteocytes. However, hypertrophic chondrocytes were negative, unlike mesenchymal cells, adjacent to the osteogenesis. Megakaryocytes and some capillary blood vessels cells were receptor positive. All ER-β expression was blocked totally by preincubation of antibody with antigen. We conclude that ER-β is expressed in cells of osteoblast lineage and in osteoclasts. The latter appear relatively abundant in this receptor and this might provide a means for direct action of estrogen on osteoclasts. (J Bone Miner Res 2001;16:214–220)

Key words: estrogen receptor β, osteoclasts, osteoblasts, chondrocytes, indirect immunoperoxidase

INTRODUCTION

THE IMPORTANCE of estrogen (17β-estradiol) for maintaining skeletal structure and function is well recognized in women; falling estrogen levels in postmenopause are associated with bone loss and increased risk of osteoporosis. Moreover, in men who are deficient in the aromatase enzyme, because of gene mutation, there is failure of epiphyseal fusion and severe osteopenia,[1,2] and falling estrogen levels in aging men are associated with bone loss.[3,4] Therefore, estrogen may be important to the skeletal development and maintenance of bone mass in men as well as women. Estrogen exerts its effects on target cells by interaction with specific estrogen receptors (ERs) of which there are now known to be at least two: ER-α, which is expressed in classic estrogen target cells, for example, in reproductive tissues, and the newly described ER-β,[5,6] the presence of which accounts for the estrogen responsiveness of some tissues, despite their low ER-α expression.[7] Thus, prostate,[8–10] ovary,[11,12] vascular cells,[13] and astro-

[1]Musculoskeletal Research Group, University of Manchester Medical School, Manchester, UK.
[2]Medical Research Council Human Reproductive Sciences Unit, Edinburgh, UK.

cytes[14] are all positive for ER-β. Furthermore, there is now increasing evidence that tissues may express both receptors, either localized to different cell types[8,14] or to the same cell.[15]

However, it is unclear how the two forms of the receptor participate in the mechanism by which estrogen acts on bone. ER-α is expressed in human cells of the osteoblast lineage both in vitro[16,17] and in vivo.[18–21] There are indications that this receptor is important to the action of estrogen on the skeleton: epiphyses remain open and there is marked bone loss in an adult man with a null mutation in ER-α[22] and expression of the receptor protein is lost from osteoblasts and osteocytes in some cases of idiopathic osteoporosis in men.[23,24] Nevertheless, although there is some reduction in bone mineral density (BMD) of ER-α knockout mice,[25] ovariectomy of these animals produced a more profound bone loss,[26] implying that other receptors may be important for estrogen action on the mouse skeleton. Indeed, there is evidence for ER-β expression in rat bone.[27,28] However, skeletal abnormalities in ER-β knockout mice are surprisingly subtle in that the bone mineral content of the cortices are increased only in adult females; males and immature animals remain unaffected.[29] The question therefore remains as to the role of ER-β in estrogen action on the skeleton, especially in humans. It is expressed in transformed fetal human cells of osteoblast lineage in vitro[30] and in growth plate of adolescent boys and girls,[31] but the exact cellular localization of ER-β in adult human bone is still unclear. Therefore, the aim of the present study was to use a recently developed polyclonal antibody specific for ER-β to localize expression of the receptor protein by immunoperoxidase. Sections of bone were included from adults with high bone turnover, to ensure the presence of a variety of bone cells. We were thus able to show clear nuclear localization of the receptor not only to cells of osteoblast lineage but also in osteoclasts.

MATERIALS AND METHODS

Patients and biopsy specimens

Biopsy specimens from men with benign hyperplasia of the prostate were used as positive control tissue throughout the study. Bone biopsy specimens used in this investigation were obtained from both men and women; fracture callus was from patients between 21 and 74 years of age, as part of normal surgical procedures, pagetic bone and osteophytes were from patients between 60 and 70 years of age, and normal bone samples were from grafts used for maxillofacial repair after trauma in patients between 40 and 75 years of age. Bone also was obtained from patients with hyperparathyroidism secondary to renal disease (50–70 years; $n = 2$ for each type of biopsy). Approval had been given by the local Research Ethics Committee, and informed consent was obtained from the patients. All samples were fixed in 10% neutral buffered formalin, the bone biopsy specimens were demineralized in 20% EDTA until radiologically decalcified (2–4 weeks), and then all biopsy specimens were processed into paraffin wax. Sections (5 μm) were cut and mounted onto slides coated with 3-aminopropyltriethyoxysilane (Sigma Chemical Co., Poole, UK).

Preparation of polyclonal antibody to ER-β

A peptide directed against the hinge (D) domain of human ER-β[5] (CAGKAKRSGGHAPRVREL) was synthesized by Affinity Reagents (Exeter, UK) and conjugated via the terminal cysteine residue to keyhole limpet hemocyanin and injected into a sheep; immunizations and recovery of antisera were all carried out by Diagnostics Scotland (Carluke, Lanarkshire, UK). Antibodies specific for ER-β were affinity purified as described in detail in Saunders et al.[32] On Western blots the purified antibodies bound to recombinant ER-β but not ER-α, and immunoreactivity in control tissues (rat epididymis) was abolished by incubation with recombinant ER-β or immunising peptide, but not ER-α.[32,33]

Immunocytochemistry

Sections were dewaxed in xylene, brought to absolute alcohol, and transferred to methanol containing 3% (vol/vol) H_2O_2 for 30 minutes to block endogenous peroxidase. Antigen retrieval was then performed by placing slides in 0.05 M glycine, containing 0.01% EDTA, pH 3.5, and heating them for 5 minutes under full pressure in a stainless steel pressure cooker (Tefal, Slough, Berkshire UK) and leaving the slides in hot buffer for a another 20 minutes. Sections were cooled, rinsed in Tris-buffered saline (TBS), after which nonspecific binding was blocked with 20% (vol/vol) normal rabbit serum (Dako, Ltd., Ely Cambridgeshire, UK) in 5% bovine serum albumin (BSA), made up in TBS, for 1 h at room temperature. They were then incubated with the antibody to ER-β diluted to 1:500 (for sections of benign hyperplasia of the prostate), 1:250, or 1:100 (for sections of bone/cartilage biopsy specimens) with the rabbit serum and BSA in TBS. Stock concentrations of the polyclonal antibody were at a concentration of 1.2 μg protein/μl. For some control experiments, the neat antibody was either preadsorbed overnight at 4°C by a 10-fold protein concentration of the immunizing antigen or left in contact with an equivalent volume of TBS, under identical conditions. Both were then diluted to 1:500 for benign hyperplasia of the prostate or 1:250 or 1:100 for bone, and sections remained in contact with antibody overnight at 4°C. In all experiments, control sections were included, which had been incubated with equivalent concentrations of nonimmune sheep immunoglobulin G (IgG; Sigma Chemical Co.). Thereafter, sections were incubated with biotinylated rabbit antibodies raised against sheep IgG (Vector Laboratories, Peterborough, UK), diluted 1:500 (vol/vol) with the rabbit serum and BSA in TBS, for 60 minutes at room temperature. Primary antibody binding to ER-β was then disclosed by standard avidin-biotin-peroxidase (Dako) followed by incubation with liquid diaminobenzidine (Sigma Chemical Co.). Sections were counterstained with hematoxylin, dehydrated, cleared, and mounted in XAM (BDH-Merk, Poole Dorset, UK).

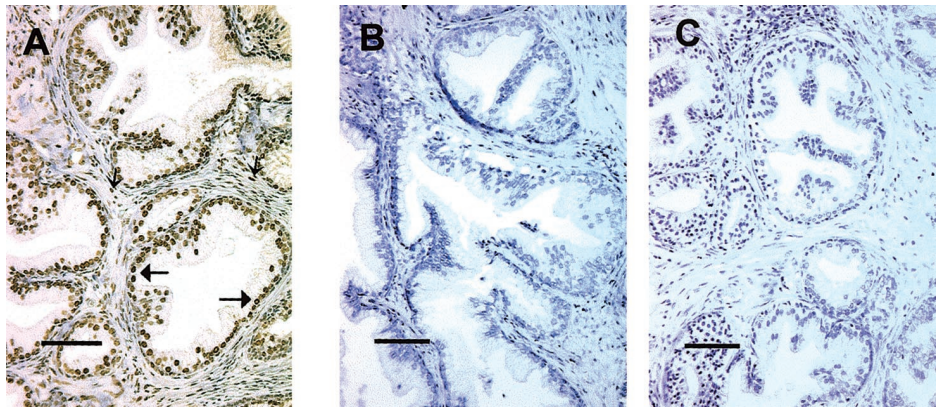

FIG. 1. ER-β expression in sections of benign hyperplasia of the prostate. Receptor was detected by immunoperoxidase, using the polyclonal antibody, as described in the Materials and Methods section at a dilution of 1:500. (A) Immunoreactivity is localized to the nuclei of glandular epithelial cells (solid arrows) and some hyperplastic cells. Some stromal cells also expressed ER-β (open arrows). When the antibodies were (B) preadsorbed with immunizing peptide, immunoreactivity was blocked completely and sections appeared similar to (C) sheep IgG control (bar = 66 μm).

RESULTS

ER-β expression in benign hyperplasia of the prostate

Localization of ER-β in sections of benign hyperplasia of the prostate is shown in Fig. 1. Retrieval of the receptor by heating under pressure in acid conditions, as described in the Materials and Methods section, was found to be essential for its detection in sections of both the positive control tissue and the bone. A primary antibody dilution of 1:500 was optimal for benign hyperplasia of the prostate, which was used as a positive control throughout this study. Immunoreactivity was localized clearly to the nuclei of epithelial cells in hyperplastic glands, although there were far fewer ER-β positive cells in the stroma. After preincubation of antibody with immunizing peptide (Fig. 1B), sections were devoid of reaction product and were similar to IgG controls (Fig. 1C).

ER-β expression in intramembraneous and endochondral bone

Osteoblasts in general were positive for ER-β, and examples are shown of these cells from focal areas of intramembraneous ossification in fracture callus (Figs. 2A and 2B) and in bone formation in osteophyte sections (Fig. 3C). Some but not all osteocytes, especially within newly formed bone, expressed ER-β (Fig. 2B). Immunoreactivity was localized to cell nuclei and was similar in bone from both men and women. Immunoreactivity was not seen in hypertrophic cartilage cells, within regions of endochondral ossification in either fracture callus (Figs. 2C and 3A) or osteophyte sections. In contrast, the adjacent mesenchymal cells and some cells entrapped within the mineralized cartilage were clearly ER-β positive, as were some of the cells within small blood vessels adjacent to the cartilage (Fig. 2D). Megakaryocytes within the marrow (Fig. 2E) also expressed the receptor. Preincubation of the antibody with ER-β completely blocked reaction product in all these cells (Fig. 2F).

ER-β expression in osteoclasts

These results are summarized in Fig. 3. Immunocytochemistry with the polyclonal antibody showed ER-β expression in osteoclast nuclei in sections of fracture callus, osteophytes, pagetic bone, and normal bone. Most osteoclasts expressed the receptor, although in some cells, not all the nuclei were positive (Fig. 3B). Little reaction product was seen in the cytoplasm of the osteoclasts. Immunoreactivity was clearly visible in osteoclast nuclei even when the polyclonal antibody was diluted to 1:500, in contrast to the other bone cells in which staining decreased when antibody was diluted more than 1:250 (data not shown). Osteoclast ER-β expression was similar in bone from men and women. Preincubation of the antibody with ER-β completely blocked immunoreactivity in osteoclasts (Fig. 3E), which was similar to IgG controls (Fig. 3F).

DISCUSSION

The study we present here shows that osteoclasts and cells of osteoblast lineage clearly express ER-β protein. That the polyclonal antibody used in the study was specific for this isoform of ER is further confirmed by the results of preadsorption with antigen, recombinant protein, and by Western blotting.[33] In our investigations and those of other researchers using fixed tissues[12,34,35] antigen retrieval by heating was found to be essential for detection of ER-β, which was accomplished with relatively low concentrations of primary antibody, compared with those used in other investigations, in which similar retrieval systems were not used.[31,36] Although the use of heating, either under pressure or by microwave, can damage sections containing bone

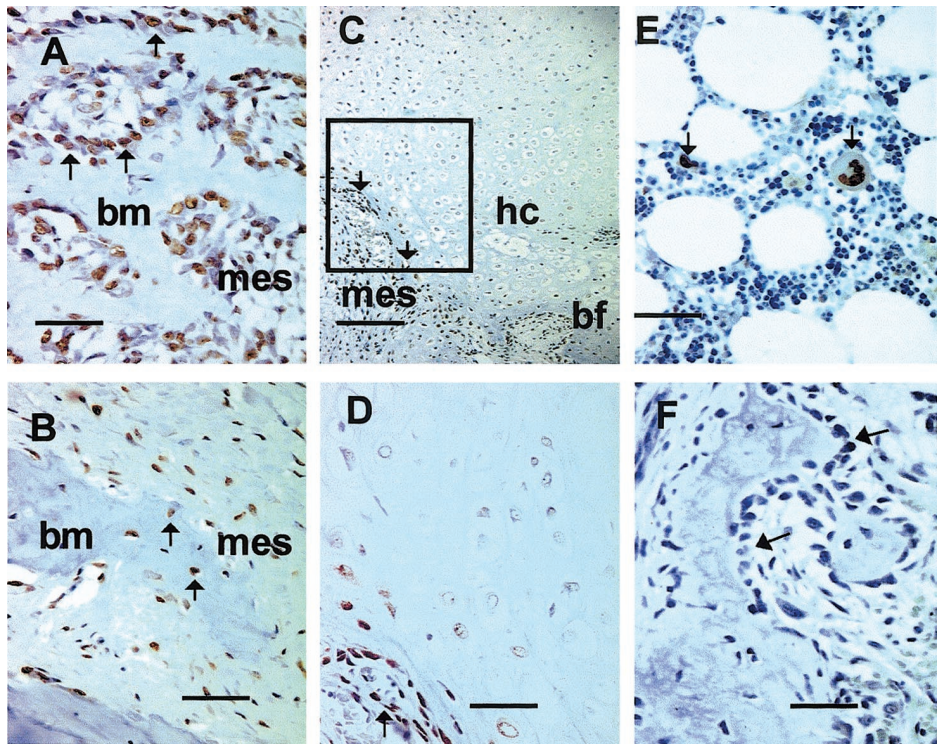

FIG. 2. ER-β expression in osteoblasts and chondrocytes. ER-β was localized by immunoperoxidase with a 1:250 dilution of the polyclonal antibody, as described in the Materials and Methods section. (A) A region of intramembraneous ossification in fracture callus from a 28-year-old man. Osteoblasts on the surface of the bone matrix (bm) are positive for the receptor; examples are shown by solid arrows, and immunoreactivity is localized to nuclei. (B) Mesenchymal cells (mes) near active bone formation also are ER-β positive, as are some osteocytes within the bone matrix, examples of which are shown by solid arrows (bar for A and B = 32 μm). (C) An area of endochondral ossification from fracture callus. Cells in the hypertrophic cartilage (hc) are negative, but those involved in endochondral bone formation (bf) or in the mesenchyme (mes), adjacent to the initial stages of endochondral osteogenesis, are ER-β positive (bar = 115 μm). (D) The inset is shown at higher magnification (bar = 32 μm). Hypertrophic cartilage cells within the cartilage are negative, but those at the perichondral edge and some of the cells in the small blood vessel wall (solid arrow) are positive for receptor. (E) ER-β is localized to nuclei of megakaryocytes (solid arrows) in a patient with secondary hyperparathyroidism. (F) Preadsorption of the polyclonal antibody with antigen blocks immunoreactivity from all cells in fracture callus; examples of osteoblasts are shown by solid arrows (bar for E and F = 32 μm).

and cartilage, we found that this was offset by the maximal exposure of receptor epitopes.

Our results show that unequivocal expression of ER-β in nuclei of osteoclasts of both men and women was positive for the receptor. The nuclear osteoclast receptor could be detected with a lower concentration of antibody than in the other bone cells, suggesting that osteoclasts have a relative abundance of ER-β. Although there are reports that estrogen regulates osteoclast differentiation indirectly, through other cell types,[37] there also is evidence that the hormone can affect mature osteoclasts directly.[38–41] Whether osteoclasts express ER-α is controversial; some groups report receptor expression either of messenger RNA (mRNA) or protein in mature cells,[19,39,42] but others show its expression in immature osteoclasts but not in the mature cells.[21,43,44] Furthermore, in a recent study of ER-β mRNA expression, using in situ hybridization, expression of the receptor could not be localized equivocally to osteoclasts in neonatal rat bone,[28] although it is unclear if many suitable cells were present in that study. However, osteoclasts, were readily visible in the biopsy specimens used in our investigation and the clear expression of ER-β protein in nuclei of mature human osteoclasts in vivo raises the possibility that estrogen may act on these cells directly.

Osteoblasts were positive for ER-β in both endochondral and intramembraneous bone formation and in areas of remodeling in adult bone. We and others have previously reported ER-α expression in cells of osteoblast lineage,[19–21,45,46] although the studies from our laboratory indicated that only a proportion of osteoblasts in bone from adult women were positive for this receptor.[20] However, from our present results (Figs. 3A and 3B) ER-β expression appears to be widespread in mature osteoblasts on bone surfaces. Some osteocytes also were positive for the receptor, as we had found previously with ER-α.[18,20]

We also observed that layers of mesenchymal cells, in healing fractures, or stromal cells, in other biopsy specimens, were strongly positive for ER-β when adjacent to sites of new bone formation, in agreement with a previous report that receptor expression was localized to ossifying zones of fracture callus.[36] In this regard, it is noteworthy that Arts et al.[30] found expression of ER-β, rather than ER-α, in cultured human fetal osteoblasts increased over the later stages of cellular differentiation up to mineralization.

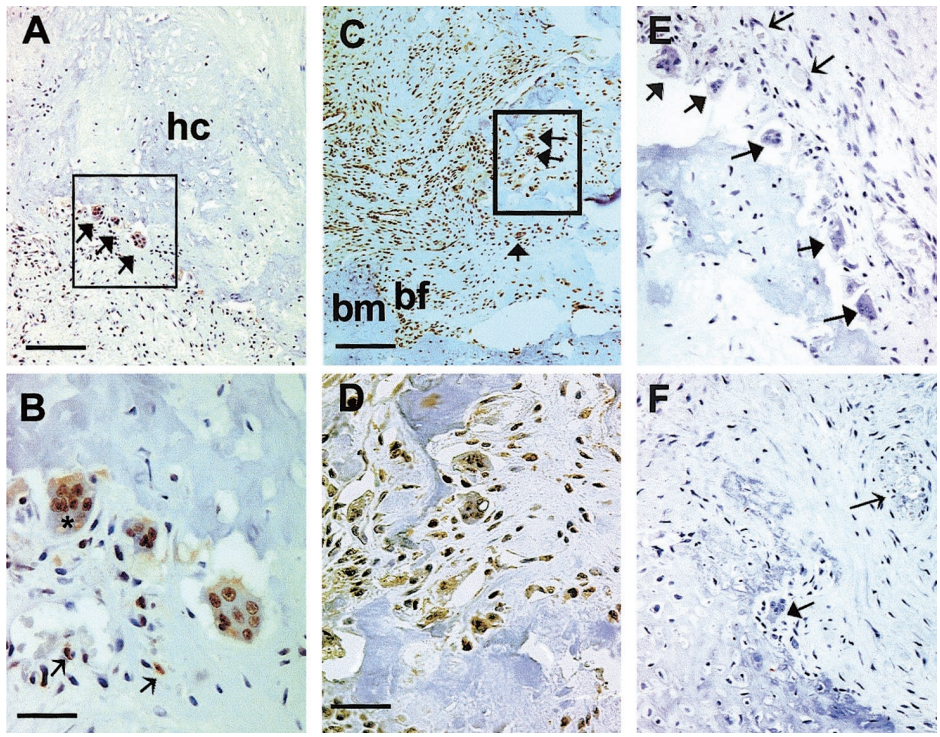

FIG. 3. ER-β expression in osteoclasts. ER-β was localized by indirect immunoperoxidase as described in the Materials and Methods section. (A) Osteoclasts, positive for ER-β (shown by solid arrows), are seen adjacent to mineralizing cartilage, undergoing endochondral ossification in fracture callus from a 21-year-old woman. Note the absence of immunoreactivity in the hypertrophic chondrocytes (hc; bar = 115 μm). (B) The inset area is shown at higher magnification (bar = 32 μm), in which ER-β expression is seen clearly in osteoclast nuclei, although there are some that are negative (*). Note some mononuclear cells nearby also are positive for the receptor (open arrows). (C) Likewise, ER-β is expressed in osteoclasts (shown by solid arrows) in sections of osteophyte from a man aged 67 years (bar = 115 μm). (D) The inset is shown at higher magnification (bar = 32 μm). In panel C cells within the bone matrix (bm) and on bone forming surfaces (bf) are positive for the receptor. (E) Immunoreactivity is blocked completely by preadsorbing the polyclonal antibody with the receptor; a facture callus section treated in this manner is shown and (F) an IgG control is shown (bar = 66 μm).

Whether the same cells express both receptors is a subject of current investigation in our laboratory. If this were so, it could provide an opportunity for heterodimerization of two isoforms[47,48] and, thereby, amplify the effects of estrogen on the bone cells. This might be especially important for men because their estrogen concentrations are low. Alternatively, if ER-α and ER-β are expressed similarly in different cells of the osteoblast lineage, even low estrogen concentrations could influence a wide spectrum of gene expression and thereby a variety of biological effects. A similar situation has been discussed for neural tissue[49] and might support a wide distribution of ER-β in the skeleton.

Distribution of ER-β expression in cartilage appeared to be localized specifically. Hypertrophic chondrocytes, in fracture callus, were largely negative, although cells within centers of endochondral bone formation were positive for the receptor. However, in growth plate from adolescent girls, hypertrophic chondrocytes are reported to express ER-β but proliferative and resting cartilage cells are negative.[31] Whether hypertrophic chondrocytes have different ER-β expression when in growth plate compared with fracture callus, or whether the different antibodies used in the two studies influence detection of this receptor, is uncertain. Nevertheless, growth plate chondrocytes in both young humans[21] and animals[50] also express ER-α. Overall, these results indicate that ER-α and ER-β are expressed either by the same or different cell types within sites of endochondral ossification, which are therefore likely to be important targets for estrogen action.

We also found ER-β protein expression in megakaryocytes within bone marrow, in agreement with a preliminary report from Khetawat et al.,[51] and in capillary blood vessels. Lindner and colleagues have described ER-β mRNA expression in the aortic endothelium and smooth muscle of male rats, both in vivo and in vitro,[13] although Baker et al. were unable to detect mRNA or protein for either ER-α or ER-β in human umbilical veins.[52] The presence of ER-β in small capillary vessels and in the megakaryocytes implies that they may respond to estrogen, which thus may be able to exert additional influences on bone through these cells.

Overall, in the cells that were positive for ER-β, we found that immunoreactivity was expressed in nuclei. Although others have reported expression of this receptor in osteoclasts,[36] they found that it was localized to the cytoplasm and not nuclei of these cells. It is uncertain whether this is because of differences in immunoperoxidase technique or of the type of biopsy specimens studied. Our investigation focused on bone from adults, in which we found no obvious

differences in ER-β expression in men compared with that in women. Furthermore, we observed strong immunoreactivity for the receptor even in bones from older patients, that is, over 60 years of age. However, in most biopsy specimens there was high bone turnover and the relation of this to ER-β expression may be an important feature of its regulation in bone.

ACKNOWLEDGMENTS

We thank Action Research for supporting this study (project grant A/P/0727).

REFERENCES

1. Morishima A, Grumbach MM, Simpson ER, Fisher C, Qin K 1995 Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological-role of estrogens. J Clini Endocrinol Metab **80:**3689–3698.
2. Morishima A, Grumbach MM, Bilezikian JP 1997 Estrogen markedly increases bone mass in an estrogen deficient young man with aromatase deficiency. J Bone Miner Res **12:**S1; S126.
3. Khosla S, Melton LJ, Atkinson EJ, OFallon WM, Klee GG, Riggs BL 1998 Relationship of serum sex steroid levels and bone turnover markers with bone mineral density in men and women: A key role for bioavailable estrogen. J Clin Endocrinol Metab **83:**2266–2274.
4. Riggs BL, Khosla S, Melton LJ 1998 A unitary model for involutional osteoporosis: Estrogen deficiency causes both type I and type II osteoporosis in postmenopausal women and contributes to bone loss in aging men. J Bone Miner Res **13:**763–773.
5. Mosselman S, Polman J, Dijkema R 1996 ER beta: Identification and characterization of a novel human estrogen receptor. FEBS Lett **392:**49–53.
6. Kuiper GGJM, Enmark E, PeltoHuikko M, Nilsson S, Gustafsson JA 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA **93:**5925–5930.
7. Gustafsson JA 1997 Estrogen receptor beta—getting in on the action? Nat Med **3:**493–494.
8. Pelletier G, LuuThe V, Charbonneau A, Labrie F 1999 Cellular localization of estrogen receptor beta messenger ribonucleic acid in cynomolgus monkey reproductive organs. Biol Reprod **61:**1249–1255.
9. Petersen DN, Tkalcevic GT, Koza-Taylor PH, Turi TG, Brown TA 1998 Identification of estrogen receptor beta(2), a functional variant of estrogen receptor beta expressed in normal rat tissues. Endocrinology **139:**1082–1092.
10. Prins GS, Marmer M, Woodham C, Chang W, Kuiper G, Gustafsson JA, Birch L 1998 Estrogen receptor-beta messenger ribonucleic acid ontogeny in the prostate of normal and neonatally estrogenized rats. Endocrinology **139:**874–883.
11. Fitzpatrick SL, Funkhouser JM, Sindoni DM, Stevis PE, Deecher DC, Bapat AR, Merchenthaler I, Frail DE 1999 Expression of estrogen receptor-beta protein in rodent ovary. Endocrinology **140:**2581–2591.
12. Sar M, Welsch F 1999 Differential expression of estrogen receptor-beta and estrogen receptor-alpha in the rat ovary. Endocrinology **140:**963–971.
13. Lindner V, Kim SK, Karas RH, Kuiper GGJM, Gustafsson JA, Mendelsohn ME 1998 Increased expression of estrogen receptor-beta mRNA in male blood vessels after vascular injury. Circ Res **83:**224–229.
14. Azcoitia I, Sierra A, GarciaSegura LM 1999 Localization of estrogen receptor beta-immunoreactivity in astrocytes of the adult rat brain. Glia **26:**260–267.
15. Shughrue PJ, Scrimo PJ, Merchenthaler I 1998 Evidence for the colocalization of estrogen receptor-beta mRNA and estrogen receptor-alpha immunoreactivity in neurons of the rat forebrain. Endocrinology **139:**5267–5270.
16. Komm BS, Terpening CM, Benz DJ, Graeme KA, Gallegos A, Korc M, Greene GL, Omalley BW, Haussler MR 1988 Estrogen binding, receptor messenger-RNA, and biologic response in osteoblast-like osteosarcoma cells. Science **241:**81–84.
17. Benz DJ, Haussler MR, Komm BS 1991 Estrogen binding and estrogenic responses in normal human osteoblast- like cells. J Bone Miner Res **6:**531–541.
18. Braidman IP, Davenport LK, Carter DH, Selby PL, Mawer EB, Freemont AJ 1995 Preliminary in situ identification of estrogen target-cells in bone. J Bone Miner Res **10:**74–80.
19. Hoyland JA, Mee AP, Baird P, Braidman IP, Mawer EB, Freemont AJ 1997 Demonstration of estrogen receptor mRNA in bone using in situ reverse-transcriptase polymerase chain reaction. Bone **20:**87–92.
20. Hoyland JA, Baris C, Wood L, Baird P, Selby PL, Freemont AJ, Braidman IP 1999 Effect of ovarian steroid deficiency on oestrogen receptor alpha expression in bone. J Pathol **188:**294–303.
21. Kusec V, Virdi AS, Prince R, Triffitt JT 1998 Localization of estrogen receptor-alpha in human and rabbit skeletal tissues. J Clin Endocrinol Metab **83:**2421–2428.
22. Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med **331:**1056–1061.
23. Braidman I, Baris C, Wood L, Selby P, Adams J, Freemont A, Hoyland JA 2000 Preliminary evidence for impaired estrogen receptor alpha protein expression in osteoblasts and osteocytes from men with idiopathic osteoporosis. Bone **26:**423–427.
24. Braidman IP, Baris C, Selby PL, Adams JA, Freemont AJ, Hoyland JA 2000 Preliminary report of impaired bone oestrogen receptor-alpha expression in bone, but no involvement of androgen receptor, in male idiopathic osteoporosis. J Pathol **192:**90–96.
25. Taki M, Kimbro KS, Washburn TF, Kiyokawa A, Okada F, Fukuda C, Miyamoto M, Korach KS 1997 Effects of estrogen receptor destruction on femurs from male mice. J Bone Miner Res **12:**S1;S460.
26. Pan LC, Ke HZ, Simmons HA, Crawford DT, ChidseyFrink KL, McCurdy SP, Schafer JR, Kimbro KS, Taki M, Korach KS, Thompson DD 1997 Estrogen receptor-alpha knockout (ERKO) mice lose trabecular and cortical bone following ovariectomy. J Bone Miner Res **12:**S1;S134.
27. Onoe Y, Miyaura C, Ohta H, Nozawa S, Suda T 1997 Expression of estrogen receptor beta in rat bone. Endocrinology **138:**4509–4512.
28. Windahl SH, Norgard M, Kuiper GGJM, Gustafsson JA, Andersson G 2000 Cellular distribution of estrogen receptor beta in neonatal rat bone. Bone **26:**117–121.
29. Windahl SH, Vidal O, Andersson G, Gustafsson JA, Ohlsson C 1999 Increased cortical bone mineral content but unchanged trabecular bone mineral density in female *ER*$\beta-/-$] mice. J Clin Invest **104:**895–901.
30. Arts J, Kuiper GGJM, Janssen JMMF, Gustafsson JA, Lowik CWGM, Pols HAP, VanLeeuwen JPTM 1997 Differential expression of estrogen receptors alpha and beta mRNA during differentiation of human osteoblast SV-HFO cells. Endocrinology **138:**5067–5070.
31. Nilsson LO, Boman A, Savendahl L, Grigelioniene G, Ohlsson C, Ritzen EM, Wroblewski J 1999 Demonstration of estrogen receptor-beta immunoreactivity in human growth plate cartilage. J Clin Endocrinol Metab **84:**370–373.

32. Saunders PTK, Millar MR, Macpherson S, Harkiss D, Anderson RA, Orr B, Groome NP, Williams K, Scobie G, Fraser HM 2000 Differential expression of estrogen receptor-alpha and -beta and androgen recpetor in the ovaries of marmoset and human. Biol Reprod **63:**1098–1105.
33. Williams K, Saunders PTK, Atanassova N, Fisher JS, Turner KJ, Millar MR, McKinnell C, Sharpe RM 2000 Induction of progesterone receptor immunoexpression in stromal tissue throughout the male reproductive tract after neonatal oestrogen treatment of rats. Mol Cell Endocrinol **164:**117–131.
34. Saunders PTK, Maguire SM, Gaughan J, Millar MR 1997 Expression of oestrogen receptor beta (ER beta) in multiple rat tissues visualised by immunohistochemistry. J Endocrinol **154:**R13–R16.
35. Saunders PTK, Fisher JS, Sharpe RM, Millar MR 1998 Expression of oestrogen receptor beta (ER beta) occurs in multiple cell types, including some germ cells, in the rat testis. J Endocrinol **156:**R13–R17.
36. Vidal O, Kindblom LG, Ohlsson C 1999 Expression and localization of estrogen receptor-beta in murine and human bone. J Bone Miner Res **14:**923–929.
37. Jilka RL, Hangoc G, Girasole G, Passeri G, Williams DC, Abrams JS, Boyce B, Broxmeyer H, Manolagas SC 1992 Increased osteoclast development after estrogen loss-mediation by interleukin-6. Science **257:**88–91.
38. Oursler MJ, Osdoby P, Pyfferoen J, Riggs BL, Spelsberg TC 1991 Avian osteoclasts as estrogen target-cells. Proc Natl Acad Sci USA **88:**6613–6617.
39. Mano H, Yuasa T, Kameda T, Miyazawa K, Nakamaru Y, Shiokawa M, Mori Y, Yamada T, Miyata K, Shindo H, Azuma H, Hakeda Y, Kumegawa M 1996 Mammalian mature osteoclasts as estrogen target cells. Biochem Biophys Res Commun **223:**637–642.
40. Robinson JA, Riggs BL, Spelsberg TC, Oursler MJ 1996 Osteoclasts and transforming growth factor-beta: Estrogen-mediated isoform-specific regulation of production. Endocrinology **137:**615–621.
41. Kameda T, Mano H, Yuasa T, Mori Y, Miyazawa K, Shiokawa M, Nakamaru Y, Hiroi E, Hiura K, Kameda A, Yang NN, Hakeda Y, Kumegawa M 1997 Estrogen inhibits bone resorption by directly inducing apoptosis of the bone-resorbing osteoclasts. J Exp Med **186:**489–495.
42. Pederson L, Kremer M, Foged NT, Winding B, Ritchie C, Fitzpatrick LA, Oursler MJ 1997 Evidence of a correlation of estrogen receptor level and avian osteoclast estrogen responsiveness. J Bone Miner Res **12:**742–752.
43. Huang WH, Lau ATT, Daniels LL, Fujii H, Seydel U, Wood DJ, Papadimitriou JM, Zheng MH 1998 Detection of estrogen receptor alpha, carbonic anhydrase II and tartrate-resistant acid phosphatase mRNAs in putative mononuclear osteoclast precursor cells of neonatal rats by fluorescence in situ hybridization. J Mol Endocrinol **20:**211–219.
44. Oreffo ROC, Kusec V, Virdi AS, Flanagan AM, Grano M, ZamboninZallone A, Triffitt JT 1999 Expression of estrogen receptor-alpha in cells of the osteoclastic lineage. Histochem Cell Biol **111:**125–133.
45. Sutherland MK, Hui DU, Rao LG, Wylie JN, Murray TM 1996 Immunohistochemical localization of the estrogen receptor in human osteoblastic SaOS-2 cells: Association of receptor levels with alkaline phosphatase activity. Bone **18:**361–369.
46. Bodine PVN, Henderson RA, Green J, Aronow M, Owen T, Stein GS, Lian JB, Komm BS 1998 Estrogen receptor-alpha is developmentally regulated during osteoblast differentiation and contributes lo selective responsiveness of gene expression. Endocrinology **139:**2048–2057.
47. Cowley SM, Hoare S, Mosselman S, Parker MG 1997 Estrogen receptors alpha and beta form heterodimers on DNA. J Biol Chem **272:**19858–19862.
48. Ogawa S, Inoue S, Watanabe T, Hiroi H, Orimo A, Hosoi T, Ouchi Y, Muramatsu M 1998 The complete primary structure of human estrogen receptor beta (hER beta) and its heterodimerization with ER alpha in vivo and in vitro. Biochem Biophys Res Commun **243:**122–126.
49. Kuiper GGJM, Shughrue PJ, Merchenthaler I, Gustafsson JA 1998 The estrogen receptor beta subtype: A novel mediator of estrogen action in neuroendocrine systems. Front Neuroendocrinol **19:**253–286.
50. Kennedy J, Baris C, Hoyland JA, Selby PL, Freemont AJ, Braidman IP 1999 Immunofluorescent localization of estrogen receptor-alpha in growth plates of rabbits, but not in rats, at sexual maturity. Bone **24:**9–16.
51. Khetawat G, Faraday N, Nealen M, Noga S, Bray PF 2000 Human megakaryocytes and platelets contain the estrogen receptor beta and androgen receptor (AR): Testosterone regulates AR expression. Blood **95:**2289–2296.
52. Baker VL, Chao VA, Murai JT, Zaloudek CJ, Taylor RN 1997 Human umbilical vessels and cultured umbilical vein endothelial and smooth muscle cells lack detectable protein and mRNA encoding estrogen receptors. J Soc Gynecol Invest **4:**316–324.

Address reprint requests to:
Dr. Isobel Braidman
Musculoskeletal Research Group
First Floor Stopford Building
University of Manchester Medical School
Oxford Road
Manchester M13 9PT, UK

Received in original form March 28, 2000; in revised form August 9, 2000; accepted October 6, 2000.