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Increased Bone Morphogenetic Protein-6 Expression in Mouse Long Bones After Estrogen Administration

ANDREA PLANT and JONATHAN H. TOBIAS

ABSTRACT

High-dose estrogen administration is known to induce new bone formation in mouse long bones. To study the role of regulatory proteins in this response, we examined associated changes in femoral messenger RNA (mRNA) for candidate factors. 17β-estradiol (E_2) 0.5 mg was administered to intact female mice by weekly injection, and Northern blot analysis was performed 1, 2, 4, 8, 12, and 16 days after the first injection. In contrast to other factors, an increase was observed in mRNA for bone morphogenetic protein-6 (BMP-6), which reached significance at day 8 and subsequent time-points. Estrogen-induced changes in BMP-6 protein expression were assessed by immunocytochemistry in longitudinal femoral sections. In untreated animals, BMP-6 was expressed by a significant proportion of growth plate chondrocytes and a subpopulation of bone marrow cells. In contrast, osteoblasts were consistently BMP-6 negative. From as early as 4 days after starting estrogen, clusters of slightly elongated BMP-6–positive cells were observed within the marrow cavity; the majority were close to active bone formation surfaces. Double immunolabeling studies revealed that only approximately 10% of BMP-6–positive bone marrow cells co-expressed the osteoblast transcription factor Cbfa1 suggesting that they are largely distinct from the osteoblast precursor population generated concurrently. BMP-6–positive cells expressed neither leukocyte nor erythroid markers (CD45 and TER-119, respectively), consistent with a stromal origin. We conclude that estrogen-induced osteogenesis in female mice is associated with increased levels of BMP-6 mRNA in mouse femurs, which seems to reflect the emergence of clusters of BMP-6 positive stromal cells adjacent to active bone formation surfaces. These findings raise the possibility that BMP-6 serves as a paracrine mediator of estrogen's osteogenic action in mice. (J Bone Miner Res 2002;17:782–790)

Key words: osteoblasts, osteogenesis, RNA, immunocytochemistry, osteoblast transcription factor Cbfa1

INTRODUCTION

It is well-recognized that estrogen exerts an important protective effect on the skeleton; loss of estrogen contributes to the development of postmenopausal osteoporosis.[1,2] Estrogen's ability to prevent bone loss is thought to involve an inhibitory action on bone resorption.[3,4] In addition, recent evidence suggests that estrogen therapy stimulates osteoblast function in postmenopausal women, particularly when administered at a relatively high dose.[5,6] Though estrogen also stimulates osteoblasts in vitro, this action is restricted to certain experimental conditions,[7,8] and the physiological relevance of these observations is unclear. Animal models also exist for studying the stimulatory action of estrogen on bone formation, of which the mouse has been used most widely.[9–11] Limitations in the latter approach are that previous studies have been restricted to use of supraphysiological doses of estrogen, and a strik-

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Rheumatology Unit, University of Bristol Division of Medicine, Bristol, United Kingdom.

ing osteogenic response is observed that may be qualitatively different from that seen in other mammals treated with estrogen.[12]

We recently found that estrogen stimulates bone formation in female mice at doses within the physiological range through an estrogen receptor (ER)–dependent mechanism.[13] We subsequently used this model to study in more detail the mechanisms by which estrogen enhances osteoblast function. Inhibition of either prostaglandin or nitric oxide synthesis significantly reduced the osteogenic response to estrogen in female mice, suggesting these pathways are involved in mediating this action.[14,15] Consistent with this conclusion, estrogen-induced osteogenesis has also been reported to be impaired in mice with a deletion in the gene for endothelial nitric oxide synthase.[16] Thus, rather than stimulating osteoblasts directly, estrogen may enhance osteoblast function by inducing the release of local regulatory factors within the marrow cavity, which subsequently stimulate bone formation through a paracrine interaction.

Bone matrix contains abundant quantities of a wide range of peptide growth factors that are known to induce osteoblast proliferation and differentiation.[17] For example, bone morphogenetic proteins (BMPs) are known to induce endochondral bone formation in vivo when administered as recombinant proteins[18,19] and are thought to play important roles in skeletal development and fracture repair.[20,21] Previous studies have reported that estrogen stimulates the release of osteogenic growth factors from osteoblasts in vitro, including BMPs,[22] insulin-like growth factor I (IGF-I),[23–25] and transforming growth factor-β (TGF-β).[25–27] However, whether these serve as autocrine or paracrine mediators of estrogen's stimulatory action on bone formation in vivo has not previously been addressed. We investigated this possibility in this study by analyzing the time-course of gene expression for a range of peptide growth factors after estrogen administration in female mice, with the initial aim of identifying factor(s) up-regulated during this response.

MATERIALS AND METHODS

Animals

Eight-week-old CBA-1 intact female mice from the University of Bristol (Bristol, UK) breeding colony were allocated to weight-matched treatment groups and were administered with 17β-estradiol (E_2) in corn oil (Sigma, Poole, Dorset, UK) at 0.5 mg/animal per week by subcutaneous (sc) injection. This dose was used to induce a maximal osteogenic response based on results of our previous dose-response study.[13] Animals were killed by cervical dislocation before or 1, 2, 4, 8, 12, or 16 days after commencement of treatment. Throughout, mice received a standard diet (rat and mouse standard diet; B&K Ltd., Humberside, UK) and water ad libitum, and they were kept on a 12-h light/dark cycle. All procedures complied with the guiding principles in *Care and Use of Laboratory Animals*.

Northern blot analysis

Northern blot analysis was performed on RNA extracted from whole femurs, including bone marrow, after E_2 treatment. For each time-point, femurs were pooled from either 4 animals for preparations of total RNA in the case of TGF-β1, or up to 10 animals where polyA$^+$ RNA was extracted in the case of other growth factors with less abundant messenger RNA (mRNA). Femurs were removed immediately after death, freed from soft tissue, snap-frozen in liquid nitrogen, and subsequently ground into a fine powder. Total RNA was obtained after incubation with Trizol reagent (Life Technologies, Paisley, UK) at room temperature for 10 minutes. Where required, polyA$^+$ RNA was isolated from total RNA using the PolyATract mRNA isolation kit (Promega, Southampton, UK). Thirty micrograms of total RNA or 2–5 μg of polyA$^+$ RNA were loaded onto denaturing gels, and transcripts were separated by electrophoresis. RNA was then transferred to a nylon membrane (Perkin Elmer, Beacons Field, UK) and covalently cross-linked to the membrane by ultraviolet (UV) irradiation.

Specific transcripts were detected by hybridization with complementary DNA (cDNA) probes labeled with α-[^{32}P]deoxycytosine triphosphate (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) by random primer method. The following probes were supplied by Dr. J. Lean (St. George's Hospital Medical School, London, UK): mouse BMP-2,[28] mouse BMP-6,[28] mouse BMP-7,[29] rat TGF-β1,[30] a 0.5 kb *Sty*I/*Eco*RI fragment of rat β-fibroblast growth factor (bFGF),[31] rat IGF-I,[32] and rat IGF-II.[33] cDNAs for rat platelet-derived growth factor (PDGF)-A[34] and PDGF-B (S. P. Templeton and S. C. Sweet, GenBank, 1999) were cloned based on sequence information held in GenBank. Blots were subsequently exposed to Phosphor Screens (Version 4; Amersham Pharmacia Biotech), and the resulting signal quantified using ImageQuant (Molecular Dynamics, Little Chalfont, Bucks, UK). To correct for gel loading, blots were stripped and reprobed with mouse β-actin cDNA.[35]

Immunolocalization studies

Based on results of Northern blot analysis, we examined the effect of estrogen on the expression of BMP-2 and BMP-6 by immunocytochemistry. Animals were treated with E_2, as above, in a separate experiment, and the femurs were removed, freed from soft tissue, paraformaldehyde-fixed, and decalcified in EDTA. The diaphysis and metaphysis were separated, and the latter was paraffin-embedded after dehydration through graded alcohols. Longitudinal sections of the distal femoral metaphysis (6 μm) were subsequently cut on a Reichert-Jung 2050 microtome and air-dried on Polysine slides (BDH, Poole, Dorset, UK). Sections were dewaxed in Xylene (BDH), rehydrated, and incubated in 10% rabbit serum (NRS; Vector Laboratories, Peterborough, UK) to block nonspecific binding of secondary antibody.

Slides were incubated overnight with goat anti-human BMP-2 (clone N-14) or BMP-6 (clone N-19; Autogen Bio-

clear, Calne, Wiltshire, UK), washed, and incubated with fluorescein isothiocyanate (FITC) conjugated rabbit anti-goat immunoglobulin G (IgG; Sigma) as a secondary antibody. Slides were subsequently washed, mounted in Vectashield mountant (Vector Laboratories), and analyzed under fluorescent microscopy (Leica) equipped with an I3 filter, using a Neotech image grabber. Control sections were included where the primary antibody was omitted or after preincubation with 5-fold excess of human recombinant BMP-6 (a gift from Genetic Institute, Cambridge, MA, USA).

To study whether BMP-6 positive bone marrow cells belong to the osteogenic lineage, we analyzed whether they co-express Cbfa1. This approach was based on evidence that Cbfa1 is an essential transcription factor for osteoblast differentiation,[36,37] and our previous finding that estrogen-induced osteogenesis in mice is associated with the appearance of large numbers of Cbfa1-expressing bone marrow cells assumed to represent early osteoblast precursors.[38] Cbfa1 protein expression was studied using a histochemical approach to enhance detection of Cbfa1 immunoreactivity. Sections were processed for BMP-6 immunodetection as described above except that an alkaline phosphatase (ALP)-conjugated donkey anti-goat secondary antibody (Jackson ImmunoResearch, Luton, UK) was used. BMP-6 immunoreactivity was detected by incubation in Vector Red substrate with 1.2 mM levamisole (Sigma) to block endogenous ALP activity. Cbfa1 immunoreactivity was subsequently detected by incubation with rabbit anti-mouse Cbfa1 primary antibody (a gift from Dr. G. Karsenty, Baylor College of Medicine, Houston, TX, USA),[39] followed by biotinylated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch). Specific immunoreactivity was amplified using a Tyramide Signal Amplification kit (Perkin Elmer) and detected with dimethylaminobenzaldehyde (DAB) substrate and nickel chloride (Vector Laboratories). Sections were subsequently mounted in DPX (BDH), and the presence of BMP-6 and Cbfa1 immunoreactivity identified under light microscopy by the presence of red and black precipitates respectively.

To further characterize BMP-6–expressing cells, double immunolabeling studies were performed using rat anti-mouse CD45 (leukocytes, clone 30-F11[40]) and TER-119 (erythroid cells[41]; Pharmingen, San Diego, CA, USA). Tissue sections were processed as above, except both primary antibodies were added simultaneously overnight followed by simultaneous incubation with rabbit anti-goat IgG-FITC and Cy3 conjugated rabbit anti-rat IgG (Jackson ImmunoResearch). Whether FITC and Cy3 showed evidence of colocalization was analyzed by fluorescent microscopy using a dual red/green filter. Cy3 alone was detected using an N2 filter for red fluorescence. Control sections were included where one or both primary antibodies were omitted.

Statistical analysis

Results for Northern blot analysis were corrected for β-actin expression and expressed as a ratio to the value obtained from animals in the same experiment immediately before starting E_2. Whether E_2 treatment affected mRNA levels was assessed by one-way analysis of variance (ANOVA) and Fisher's test of least-significant difference performed where $p < 0.05$ (Statview 4.0; SAS Institute, Cary, NC, USA).

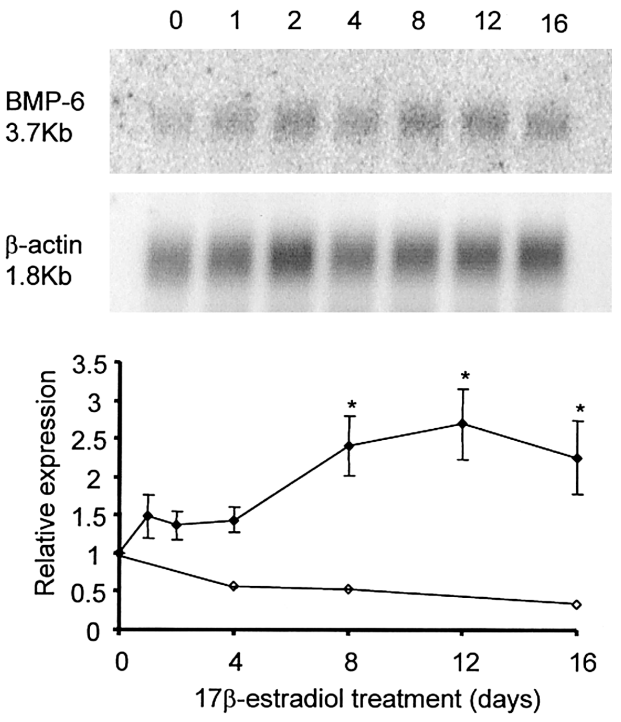

FIG. 1. Effect of treatment with 17β-estradiol (E_2), 0.5 mg/animal per week for varying durations, on BMP-6 expression of mouse femurs. Top: picture of phosphor imager screen used for mRNA quantification in a representative experiment. Bottom: mean ± SEM (closed symbols) after combining results from three independent experiments in which RNA was extracted from femurs pooled from 10 (2 experiments) or 4 (1 experiment) mice per time-point. In each experiment, results were expressed as the BMP-6/actin ratio divided by the time 0 value. Open symbols represent data from a control group of animals killed after vehicle administration during the first experiment (four animals per time-point). *$p < 0.05$ versus day 0 by one-way ANOVA.

RESULTS

E_2 treatment significantly increased BMP-6 mRNA from day 8 onwards, assessed by combining results from three independent experiments (Fig. 1). No equivalent increase was observed in control animals in which BMP-6 expression was analyzed at different time-points after vehicle treatment. Preliminary findings based on analysis of one experiment suggested that other growth factors do not show a similar increase (Table 1). Indeed, the majority of these other factors, including TGF-β1, BMP-2, and IGF-1, showed trends suggestive of reduced expression after E_2 administration.

To study estrogen-induced changes in expression of BMP-2 and BMP-6 at the protein level, immunocytochemistry was subsequently performed on longitudinal femoral

TABLE 1. EFFECT OF TREATMENT WITH 17B-ESTRADIOL (E_2) 0.5 mg/ANIMAL PER WEEK FOR VARYING DURATIONS ON GROWTH FACTOR EXPRESSION OF MOUSE FEMURS

	17β-Estradiol treatment					
Growth factor	*Day 1*	*Day 2*	*Day 4*	*Day 8*	*Day 12*	*Day 16*
TGF-β1 (2.5 kb)	0.89	0.50	0.61	0.51	1.05	1.08
BMP-2 (3.8 kb)	1.11	0.63	0.61	0.59	0.83	0.64
PDGF-A (2.0 kb)	1.13	0.79	0.78	1.49	0.95	0.85
PDGF-A (6.3 kb)	1.27	0.67	1.04	0.79	0.67	0.68
PDGF-B (3.5 kb)	0.96	0.93	1.25	1.0	0.96	1.02
IGF-I (7.0 kb)	0.50	0.70	0.75	0.89	1.12	1.03
IGF-I (1.8 kb)	0.78	0.85	0.90	0.77	0.65	0.66
IGF-I (1.0 kb)	1.07	0.43	0.42	0.49	1.00	0.85

Results show the growth factor/actin ratio divided by the time 0 value for RNA samples pooled from femurs of 10 animals per time-point.

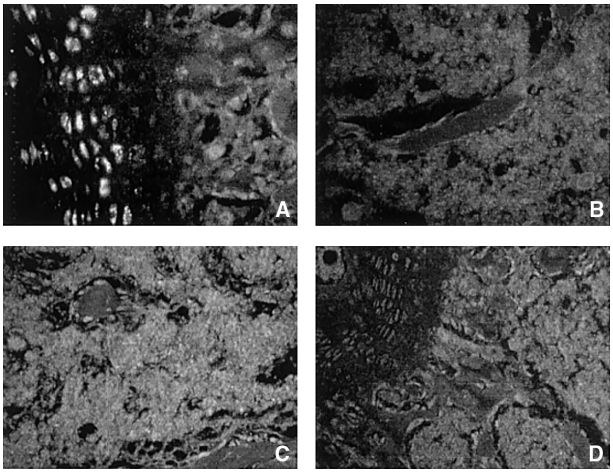

FIG. 2. Longitudinal femoral sections incubated with BMP-2 antibody and subsequently visualized by indirect immunofluorescence. Sections from untreated mice show (A) BMP-2 expression by growth plate chondrocytes (×200) and (B) lack of BMP-2 expression within bone marrow (×200). (C) Section obtained 8 days after starting E_2 0.5 mg/week, in which BMP-2 expression within bone marrow is still absent (×200). (D) Lack of immunostaining after omission of BMP-2 antibody (×200).

sections. BMP-2 expression was restricted to growth plate chondrocytes in untreated animals (Figs. 2A and 2B), which remained the case after administration of E_2 (Fig. 2C). Controls in which the primary antibody was omitted showed no immunostaining (Fig. 2D).

A significant proportion of growth plate chondrocytes were also found to express BMP-6 (Fig. 3A). Preincubation of BMP-6 antibody with recombinant human BMP-6 led to a decrease in the intensity of the BMP-6 signal, confirming the specificity of BMP-6 immunoreactivity (Fig. 3B). No immunoreactivity was observed in sections where the primary antibody was omitted (Fig. 2D). A small number of bone marrow cells were also observed to express BMP-6 (Fig. 3C). In contrast, osteoblasts consistently failed to express BMP-6. E_2 treatment seemed to reduce BMP-6 expression within the growth plate, presumably related to estrogen's inhibitory action on longitudinal bone growth (Fig. 3D).

Interestingly, E_2 led to the appearance of clusters of BMP-6–positive bone marrow cells within the metaphysis, which were first identified in sections obtained 4 days after starting E_2 (Fig. 4). By day 8, these clusters included cells with a slightly elongated morphology and were generally close to active bone formation surfaces (Figs. 3E–3G). At day 16, the marrow cavity within the metaphysis had largely been replaced by new cancellous trabeculae, and relatively few BMP-6–positive bone marrow cells were present (results not shown). In contrast, the marrow cavity of the diaphysis, which had not yet been replaced by new bone, showed large numbers of BMP-6–positive cells throughout (Fig. 3H).

We examined the relationship of BMP-6–expressing cells to the osteogenic lineage by analyzing whether they co-express the osteoblast transcription factor, Cbfa1. Several growth plate chondrocytes were identified which co-expressed BMP-6 and Cbfa1 (Fig. 4A). In untreated animals, a small number of bone marrow cells were identified that expressed Cbfa1; the occasional cell co-expressed BMP-6 (Fig. 4B). Within 4 days of E_2 treatment, clusters of BMP-6–positive bone marrow cells could be identified (Figs. 4C and 4D). Large numbers of Cbfa1-positive bone marrow cells were also present at this time, distributed relatively evenly throughout the marrow cavity, as previously observed.[38] A small number of bone marrow cells co-expressed BMP-6 and Cbfa1, representing ~10% of the BMP-6–positive bone marrow population. Sections in which one of the primary antibodies was omitted showed absent immunostaining as appropriate (Figs. 4E and 4F).

Further double immunolabeling studies were performed to examine the relationship of BMP-6–expressing bone marrow cells to other lineages. BMP-6–positive bone marrow cells expressed neither CD45 nor TER-119, suggesting that they are not of leukocyte or erythroid origin (Figs. 5A and 5B). Sections incubated with antibodies to CD45 or TER-119 alone showed the expected tissue distribution for these lineages (Figs. 5C and 5D).

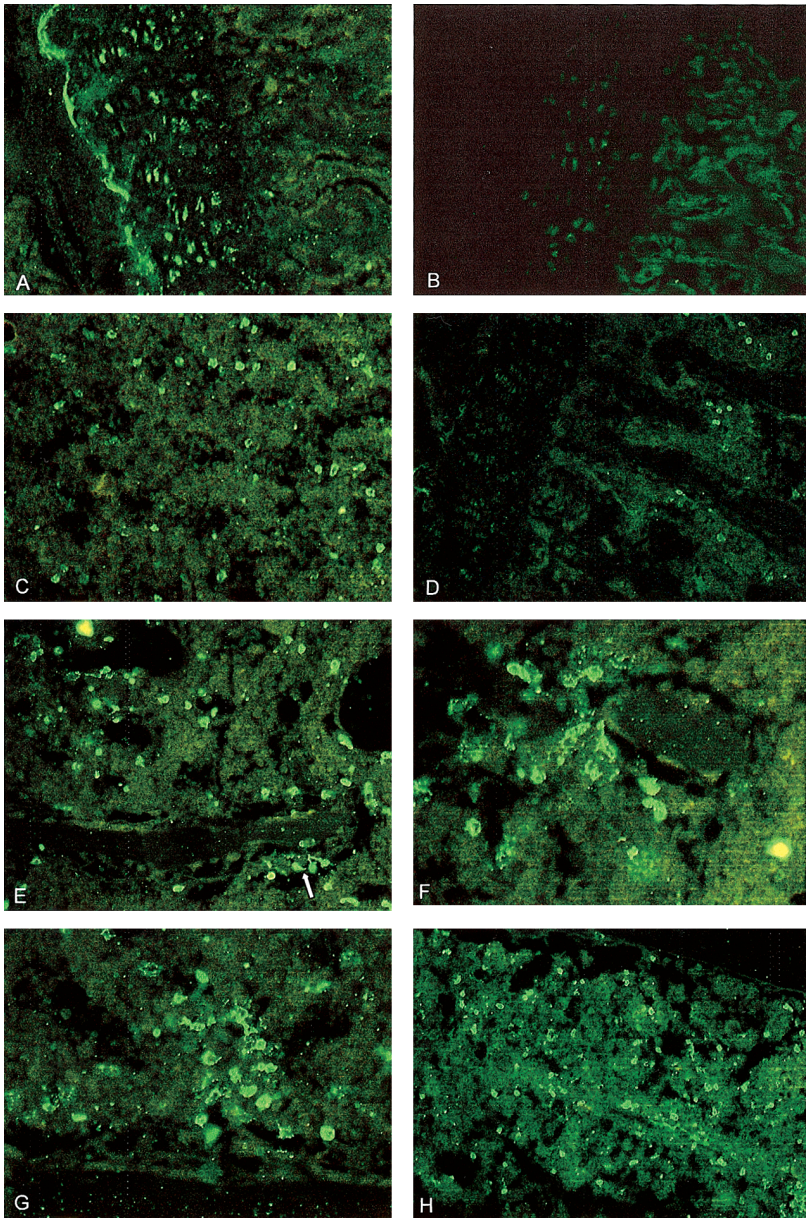

FIG. 3. Longitudinal femoral sections incubated with BMP-6 antibody and subsequently visualized by indirect immunofluorescence. Sections from untreated mice show (A) BMP-6 expression by growth plate chondrocytes (×100), (B) reduced immunostaining in the growth plate of untreated animals after preincubation with recombinant human BMP-6 (×100), and (C) BMP-6–positive cells within bone marrow (×200). Sections obtained 8 days after starting E_2 show (D) reduced BMP-6 immunostaining of the growth plate (×100), (E) clusters of BMP-6–positive cells adjacent to active bone formation surfaces (arrow; ×200), (F) BMP-6–expressing cells adjacent to cancellous bone formation surface under higher power (×400), and (G) BMP-6–positive cells adjacent to endosteal formation surface (×400). (H) Sections obtained at day 16 show large numbers of BMP-6–expressing bone marrow cells throughout the diaphysis (×200).

DISCUSSION

We investigated the mechanisms that mediate new bone formation after estrogen administration in female mice by analyzing whether this response is associated with increased expression of candidate growth factors, which were assessed by quantification of femoral mRNA. A significant rise in BMP-6 mRNA was observed on day 8 and beyond, whereas in contrast, no increase was observed in other factors analyzed. Subsequent immunocytochemistry studies revealed that estrogen treatment led to the appearance of clusters of BMP-6–positive cells within the marrow cavity as early as 4 days after starting estrogen. In view of their similar time-courses, we assume that expansion in the BMP-6–positive bone marrow population after estrogen was responsible for the rise in femoral BMP-6 mRNA.

The increase in BMP-6 expression after the addition of estrogen preceded the osteogenic response that was assessed in our previous time-course studies, performed under identical experimental conditions.[11,42] For example, type I collagen expression and the extent of mineralizing surfaces, which were assessed by Northern blot analysis and histomorphometry, respectively, were not found to increase until 12 days after starting estrogen. Because BMP-6 expression seemed to increase during the early phase of the osteogenic response to estrogen and the majority of clusters of BMP-6–positive bone marrow cells were in close proximity to sites of active bone formation, our findings raise the possibility that BMP-6 serves as a paracrine mediator of estrogen-induced bone formation. This suggestion is consistent with previous evidence that BMP-6 is a potent inducer of osteoblast differentiation both in vitro and in vivo.[43–47]

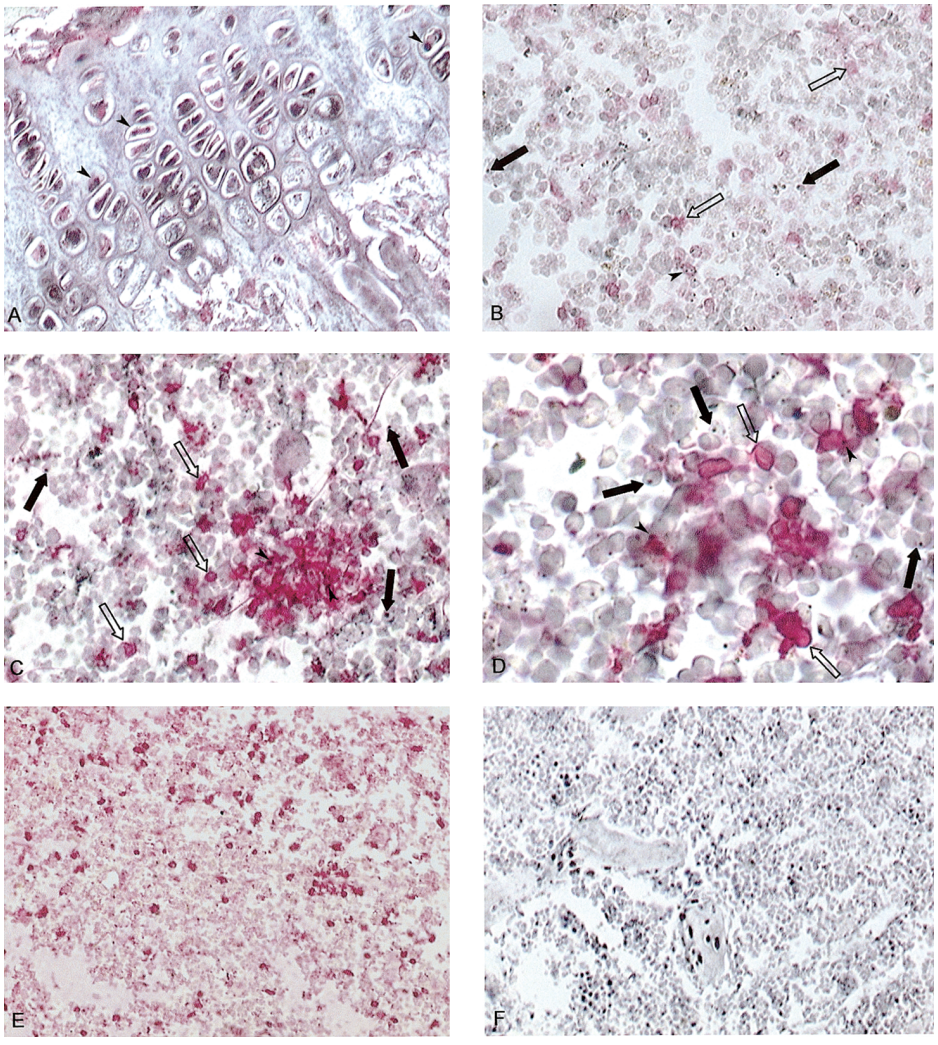

FIG. 4. Longitudinal femoral sections incubated with antibodies to BMP-6 and Cbfa1 and subsequently viewed by light microscopy. (A) In growth plate sections, the majority of chondrocytes showed high levels of Cbfa1 expression, whereas several chondrocytes were identified that co-expressed BMP-6 (arrowhead; ×200). (B) Section from an untreated mouse showing BMP-6–expressing bone marrow cells (open arrow), a small number of bone marrow cells that expressed Cbfa1 (filled arrow), and the occasional double-positive cell (arrowhead; ×200). Sections obtained 4 days after starting E_2 0.5 mg/week, under (C) lower power (×200) and (D) higher power (×400), showing clusters of BMP-6–positive cells within bone marrow and a marked increase in the number of Cbfa1-positive bone marrow cells; ~10% of BMP-6–positive cells co-expressed Cbfa1. Control sections were obtained 4 days after starting E_2 0.5 mg/week: (E) primary antibody for Cbfa1 omitted, showing BMP-6–positive cells within bone marrow (×100) and (F) primary antibody for BMP-6 omitted, showing expression of Cbfa1 by bone marrow cells and osteoblasts (×100).

The exact nature of BMP-6–positive bone marrow cells identified in this study is unclear. Although growth plate chondrocytes have previously been shown to express BMP-6,[47] we are not aware of any previous reports of BMP-6 expression in bone marrow cells. Because stromal cells regulate other bone marrow lineages and secrete a wide range of growth factors,[48] BMP-6–positive bone marrow cells may represent a stromal population involved in stimulation of osteoblast formation through a paracrine interaction. Preliminary analysis was consistent with this possibility because BMP-6–positive cells lacked markers for other marrow populations such as erythroid or leukocyte lineages. However, definitive identification of stromal cells is difficult due to the lack of specific markers.

In view of previous findings that cells of the osteogenic lineage secrete BMP-6,[22] BMP-6–positive bone marrow cells may alternatively represent early osteoblast precursors in which BMP-6 acts to stimulate further differentiation through an autocrine mechanism. Consistent with this possibility, we previously found that estrogen induces a marked increase in the population of early osteoblast precursors within bone marrow of mouse femurs, as assessed by an ex

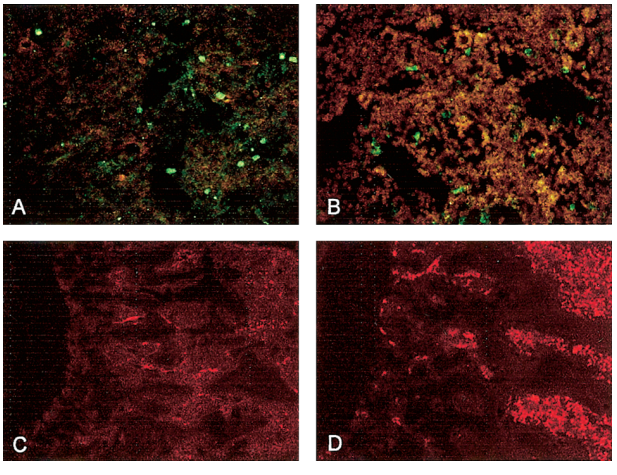

FIG. 5. Longitudinal femoral sections from untreated mice incubated with antibodies to (A) BMP-6 and CD45 or (B) TER-119 and subsequently viewed by indirect immunofluorescence (×200). BMP-6–positive bone marrow cells expressed neither CD45 nor TER-119. (C) CD45 antibody alone (×100). (D) TER-119 antibody alone (×100).

vivo CFUf assay and number of Cbfa1-positive bone marrow cells.[49,50] However, relatively few BMP-6–positive bone marrow cells seemed to co-express Cbfa1, suggesting that BMP-6–positive cells are largely distinct from the Cbfa1-positive osteoblast precursor population. Because Cbfa1 expression is restricted to the nucleus, the number of Cbfa1-positive cells may have been underestimated, because the nucleus may not have been included in the plane of section for a significant number of bone marrow cells. However, BMP-6– and Cbfa1-positive cells also showed differing distributions within bone marrow, suggesting that these represent essentially separate populations. For example, BMP-6–positive cells were initially seen to form clusters of relatively elongated cells, which was not the case for cells which expressed Cbfa1. Furthermore, large numbers of BMP-6–positive cells continued to be expressed throughout the marrow cavity as assessed up until 16 days after starting estrogen, whereas at these later time-points, Cbfa1-positive bone marrow cells are generally located adjacent to bone forming surfaces.[38]

Although our results suggest that BMP-6– and Cbfa1-positive cells represent distinct populations, it is likely that important functional relationships exist between these. For example, previous findings indicate that considerable plasticity exists in mesenchymal-derived lineages,[51] and Cbfa1 expression is necessary but not sufficient for osteoblast differentiation.[52] Hence, paracrine growth factors such as BMP-6 may contribute to bone formation by stimulating and maintaining osteogenic commitment of Cbfa1-positive early osteoblast precursors. This possibility is supported by previous evidence that BMP-6 is distinct from other BMPs because it acts principally at the level of early osteoprogenitor cells.[44]

To our knowledge, this is the first report of increased BMP-6 expression in vivo in response to administration of estrogen or any other stimulus of bone formation. Estrogen has previously been found to induce BMP-6, but not other BMPs, in fetal human osteoblast cell lines.[22] Although this observation implies that estrogen can directly induce BMP-6 expression by osteoblasts, the latter were not found to express BMP-6 in this study. Furthermore, our results suggest that estrogen is more likely to have increased expression of BMP-6 in mouse femurs by expanding the BMP-6–positive population than by direct activation of gene transcription. For example, a relatively close correlation was found between the increase in BMP-6 mRNA content after estrogen administration and the appearance of clusters of BMP-6–positive cells. In addition, any direct tendency of estrogen to induce BMP-6 gene transcription may be expected to have caused a more rapid increase in mRNA levels than observed here.

Estrogen is also thought to stimulate osteoblast function in humans[5,6] and other mammals.[53–55] In view of our findings, it is tempting to speculate that BMP-6 is involved in mediating stimulatory effects of estrogen on osteoblast function in other species. Although this study used a pharmacological dose of estrogen to induce a maximal response, it is likely that lower doses of estrogen, which we have previously found to stimulate bone formation in mice,[13] act through a similar mechanism. However, whereas BMP-6 is suggested to play a role in estrogen-induced osteogenesis in mice by stimulating early osteoblast precursors, in other species, estrogen may act predominantly to prolong osteoblast survival.[56,57] Previous clinical studies have reported that cells of the osteoblast lineage express estrogen receptors,[58,59] and that estrogen administration leads to an increase in serum levels of IGF-I.[60] However, to our knowledge, no previous study has analyzed possible influences of estrogen on expression of BMP-6 or other growth factors within the human skeleton. Our findings may also reflect a wider role of BMP-6 in bone formation. For example, the permissive effect of glucocorticoids on osteoblast differentiation has been found to be mediated by BMP-6 specifically,[45] and BMP-6 expression has been suggested to play an important osteoinductive role in osteoblastic metastases associated with prostate adenocarcinoma.[61]

Although this study focused on the role of BMP-6 in mediating estrogen-induced bone formation, other paracrine mediators are likely to be involved in this response. For example, we recently reported that estrogen-induced bone formation is partially suppressed by inhibition of prostaglandin and nitric oxide synthesis.[14,15] Though growth factors such as TGF-β1 and IGF-1 were also predicted to play a role in this response based on results of studies with isolated osteoblasts,[23–27] our preliminary findings suggested that if anything, expression of these factors tended to fall after estrogen administration. Interestingly, administration of diethylstilbestrol (DES) has also been reported to suppress IGF-I expression in rat bones.[62] However, relatively few growth factors were examined in the present investigation, and whether estrogen selectively up-regulates expression of BMP-6 as opposed to other regulatory factors is currently unclear. It would also be informative to determine whether BMP-6 is solely responsible for the increased bone formation after estrogen administration by examining whether this response is impaired in BMP-6 knockout mice.[63]

A potential limitation of this study is that growth factor mRNA expression was assessed at the level of whole bone. This approach was successful in detecting changes in expression of BMP-6 that were subsequently confirmed by immunocytochemistry. However, the lack of any overall increase in expression of other growth factors during estrogen-induced osteogenesis may have reflected the fact that several compartments were being analyzed concurrently and estrogen may have exerted opposing effects. For example, TGF-β1 is expressed by hemopoietic cells,[64] which undergo a rapid decline after high-dose estrogen administration in female mice.[49] Similarly, any tendency for BMP-2 mRNA to fall after E_2 treatment may have reflected estrogen's suppression of longitudinal bone growth, because estrogen seemed to reduce chondrocyte expression of BMP-2 to a similar extent observed for BMP-6 (results not shown).

In conclusion, we found that estrogen-induced osteogenesis in female mice is associated with increased femoral content of BMP-6 mRNA. Further analysis revealed that this change reflects the appearance of clusters of BMP-6 expressing bone marrow cells adjacent to active bone formation surfaces. These findings raise the possibility that

BMP-6 expression seems to be indirectly increased in response to estrogen administration. Further studies are required to determine whether BMP-6 may perhaps mediate the action of estrogen on the skeleton in other species including humans.

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Address reprint requests to:
J. H. Tobias, M.D., Ph.D., F.R.C.P.
Rheumatology Unit
Bristol Royal Infirmary
Bristol BS2 8HW, UK

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