IT IS WELL RECOGNIZED that estrogen exerts an important protective effect on the skeleton and that loss of this action after menopause contributes to the pathogenesis of postmenopausal osteoporosis. Although this protective action of estrogen is largely thought to be mediated by inhibition of bone resorption (3), recent evidence suggests that stimulation of osteoblast activity may also be involved, particularly at concentrations within the upper physiological range (39). To investigate the mechanisms by which estrogen stimulates osteoblast function in more detail, we utilized the mouse model. In this species, high-dose estrogen induces osteosclerosis within the shaft of long bones (9, 40) as a consequence of increased osteoblastic cellular activity (1, 32, 33). We have found that, after high-dose estrogen treatment in female mice, new sites of cancellous bone formation rapidly appear within the tibial metaphysis, presumably as a result of the generation of osteoblasts from osteoprogenitor cells within bone marrow (29).

The osteosclerosis that high-dose estrogen induces in the long bones of female mice results in a considerable reduction in the space available for hematopoiesis and is associated with the development of extramedullary hematopoiesis (21). At first sight, it would seem likely that suppression of hematopoietic marrow after high-dose estrogen is secondary to replacement of the bone marrow cavity by new bone. Alternatively, it is possible that any inhibitory effect of high-dose estrogen on hematopoietic marrow represents a primary action of this hormone, in which case the associated osteogenic response may reflect a secondary phenomenon. That osteogenesis may be a consequence rather than a cause of estrogen’s suppressive effects on hematopoiesis is consistent with previous observations that other forms of hematopoietic depletion, such as that induced by gamma irradiation, are likewise associated with osteogenesis (23).

Although important functional relationships are likely to exist between medullary hematopoiesis and osteogenesis, few in vivo studies have investigated these. In light of observations that suggest that estrogen exerts major effects on medullary hematopoiesis and osteogenesis in mice, this would appear to represent a useful experimental model for examining relationships between these two processes. In the present study, we addressed this possibility by comparing the time course of estrogen’s effects on hematopoiesis and osteogenesis in female mice.

METHODS

Mice. Ten-week-old CBA-1 female mice were obtained from the University of Bristol Medical School breeding colony. Throughout the following experiments, mice received a standard diet (Rat and Mouse Standard Diet; B & K, Humberside, UK) and water ad libitum and were kept on a 12:12-h light-dark cycle. 17β-Estradiol was dissolved in corn oil (Sigma Chemicals, Poole, Dorset, UK) and was administered by subcutaneous injection at 500 μg/wk to each animal. At the termination of the experiment, animals were killed by cervical dislocation. All experimental procedures complied with the guiding principles in the Care and Use of Laboratory Animals.

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Experiment 1. We analyzed the temporal changes in hematopoietic subpopulations of bone marrow from mouse femurs, after administration of 17β-estradiol as above. Animals were divided into seven groups (4/group) and were killed immediately before or 2, 4, 8, 12, 15, or 18 days after the first subcutaneous injection of 17β-estradiol. The left femur was removed and cleaned of tissue, and the epiphyses were removed. Bone marrow was collected by repeated flushing of the contents of the femur into a tube with 1.5 ml in 0.01 M PBS, pH 7.4, using a syringe with a 25-gauge needle until the bone appeared blanched. The bone marrow was dispersed in a cell suspension by careful use of the syringe needle against the tube wall.

A range of monoclonal antibodies was used to define populations of bone marrow cells before and after 17β-estradiol administration (see Table 1). For staining, the bone marrow cells in suspension were placed in plastic tubes at a concentration of $10^6$ cells/100 μl PBS with the appropriate antibodies (Table 1) and were incubated on ice for 30 min. In a separate tube, 100 μl of the cell suspension was added to 400 μl of PBS for subsequent accurate determination of cell numbers. Cells were washed three times (800 g at 4°C for 5 min) in PBS containing 1% (wt/vol) BSA and then were incubated for a further 30 min at 4°C with the respective second antibodies. These were R-phycoerythrin-conjugated rabbit F(ab’)2 fragment anti-rat IgG (Serotec, Oxford, UK) and R. phycoerythrin-conjugated goat F(ab’)2 fragment antimouse immunoglobulins (Dako, UK).

Flow cytometry of the fluorescently stained bone marrow cells was performed using a Becton-Dickinson FACSCAN Calibur machine. Acquisition was performed by gating the cell population on a side light scatter (cell granularity) vs. forward machine. Acquisition was performed by gating the cell population on a side light scatter (cell granularity) vs. forward light scatter (FSC; cell size) dot plot. An FSC cutoff of $>6$ μm was set up by exclusion of 6-μm calibration beads (Becton-Dickinson) before acquisition was performed. Fluorescence intensity was determined using a single-parameter histogram of cell number vs. R. phycoerythrin fluorescence. Non-specific binding (background) was determined using the appropriate isotype control antibody. The percentage of positive cells for each antibody was calculated by measuring an increase in the fluorescence intensity relative to the settings of the isotype control.

Experiment 2. We were also keen to evaluate estrogen-induced changes in the size of the megakaryocyte population. Because these cells are rare and therefore difficult to analyze with flow cytometry, we addressed this question by histology. In preliminary studies, we sought to confirm the identity of megakaryocytes as multinucleate cell profiles that were not directly opposed to a bone surface by performing immunohistochemistry on tibial sections. Acetone-fixed serial 5-μm sections were cut from snap-frozen mouse tibiae and were incubated in 1% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. After being washed in PBS, sections were incubated with 20% normal goat serum, followed by a purified rat anti-mouse CD41 monoclonal antibody, which is specific for megakaryocytes (24; clone MWRreg30; Pharmingen, San Diego, CA). Binding to CD41 was visualized using a combination of biotinylated goat anti-rat IgG antibody (Chemicon) and diaminobenzidine. Sections were counterstained with 0.25% toluidine blue.

To examine the effect of estrogen on megakaryocyte number and to relate these changes to the associated osteogenic response, mice were subsequently divided into six groups (4/group), administered 17β-estradiol as above, and killed before or 1, 2, 4, 8, or 12 days after the first subcutaneous injection. Tibiae were subsequently removed and cleared of soft tissue, fixed in 10% formal saline for 48 h, decalcified for 5 days in 5.5% EDTA, dehydrated through graded alcohols, and embedded in paraffin. Longitudinal sections of the proximal tibial metaphysis were cut on a Reichert-Jung 2050 microtome and were stained with hematoxylin and eosin.

Histological analysis of the proximal tibial metaphysis was subsequently performed using transmitted light microscopy linked to a computer-assisted image analyzer (Osteometrics, Atlanta, GA). The sampling area consisted of the entire proximal metaphysis with the proximal border situated 0.25 mm below the growth plate to exclude primary spongiosa and the distal border 3 mm distal to this (29). After confirmation in preliminary studies as above, megakaryocytes were identified as multinucleate cell profiles that were not directly opposed to a bone surface. Results were expressed as megakaryocyte number per total tissue area in millimeters squared. In addition, cancellous bone volume was analyzed as previously described, with results being expressed as a percentage of total tissue volume (29). All sections were examined blind at $\times 200$ magnification, with two nonconsecutive sections analyzed per animal.

Table 1. Antibodies used for phenotypic characterization of bone marrow cells

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Reactivity</th>
<th>Isotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>KBA</td>
<td>CD11a: lymphocyte function associated molecule-1 antigen. Expressed by all leucocytes, with the exception of some macrophages (36).</td>
<td>Rat IgG2a</td>
<td>Serotec (Oxford, UK)</td>
</tr>
<tr>
<td>RA3-6B2</td>
<td>CD45R: form of CD45 antigen expressed by B lymphocytes and subset of natural killer cells (4).</td>
<td>Rat IgG2a</td>
<td>Serotec</td>
</tr>
<tr>
<td>MOMA-2</td>
<td>Monocytes and macrophages (15).</td>
<td>Rat IgG2a</td>
<td>Serotec</td>
</tr>
<tr>
<td>MEC 13.3</td>
<td>CD31: platelet endothelial cell adhesion molecule, a 130k integral membrane glycoprotein member of the immunoglobulin superfamily involved in cell-cell adhesion. Expressed by neutrophil monocytes, endothelial cells, and some myeloid stem cells (5).</td>
<td>Rat IgG2b</td>
<td>Chemicon (Temecula, CA)</td>
</tr>
<tr>
<td>BMA5</td>
<td>VLA-5: fibronectin receptor α5β1 complex (CD49e). Expressed by monocytes, lymphocytes, fibroblasts, and endothelial cells (11).</td>
<td>Rat IgG2b</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>TER-119</td>
<td>Erythroid cells (Ly-76) from the early erythroblast through to mature erythrocyte stages (12).</td>
<td>Rat IgG2b</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>143-3</td>
<td>Alkaline phosphatase. Expressed by osteoblasts (27, 37) and granulocytes (8).</td>
<td>Mouse IgG1</td>
<td>Gift from Dr. Gideon Rodan</td>
</tr>
<tr>
<td>LO-DNP-16</td>
<td>Isotype control</td>
<td>Rat IgG2a</td>
<td>Serotec</td>
</tr>
<tr>
<td>MCA1125</td>
<td>Isotype control</td>
<td>Rat IgG2a</td>
<td>Serotec</td>
</tr>
<tr>
<td>MOPC-21</td>
<td>Isotype control</td>
<td>Mouse IgG1</td>
<td>Sigma (Poole, UK)</td>
</tr>
</tbody>
</table>
EFFECTS OF ESTROGEN ON HEMATOPOIESIS AND OSTEOGENESIS

Experiment 1. Administration of 17β-estradiol led to a gradual decline over time in the number of cells that could be flushed from each femur, which reached significance by day 18 (Fig. 1). In contrast, 17β-estradiol led to rapid reductions in the number of cells that expressed CD11a, CD31, or CD45R. The leukocyte population, as defined by expression of CD11a, showed a striking decrease between days 2 and 4. By day 18, the size of the population had declined further to ~10% of baseline levels. The population of cells defined by CD31 expression, which included hematopoietic and endothelial cells (see Table 1), displayed a minor increase in number at day 2 but then also declined significantly by day 4; the trend continued such that the population was absent altogether at day 12. Cells of the B lymphocyte lineage expressing CD45R showed a transient initial increase after estrogen treatment followed by a marked decrease, mirroring the change in overall leukocyte population as defined by the CD11a-positive fraction.

Monocytes expressing MOMA-2 showed a more gradual, but nonetheless marked, reduction in number, becoming undetectable by day 15. The very late antigen-5-positive fraction, which contains fibroblasts, endothelial cells, and some leucocytes, showed a similar decline over time to the monocyte population. In contrast, the population of erythroid lineage cells (TER-119 positive) increased in size, peaking at day 8. Cells expressing ALP, consisting predominantly of granulocytes and osteoblasts (see Table 1), initially declined in number but subsequently showed no overall change between days 4 and 18.

Experiment 2. In preliminary studies, multinucleate cells within bone marrow not directly opposed to bone surfaces were identified (Fig. 2A), all of which showed positive immunolabeling for the megakaryocyte-specific antibody CD41 (Fig. 2B). Control sections in which the primary antibody was omitted are shown in Fig. 2C. In a subsequent time course study, commencement of 17β-estradiol was followed by a transient increase in the number of megakaryocytes, with a peak increase of nearly 50% at 2 days (Fig. 2D). This was succeeded by a gradual decline in numbers such that by 12 days after starting treatment with 17β-estradiol megakaryocyte numbers were reduced by ~75%. A similar response was observed when megakaryocyte number was expressed against remaining bone marrow area (results not shown). 17β-Estradiol also increased cancellous bone volume in the tibial metaphysis, which reached significance by 12 days after starting treatment (Fig. 3).

Experiment 3. In bone marrow cultures established 8 days after starting 17β-estradiol, a 30-fold increase in osteogenic precursors was observed, as assessed by counting CFU-AP (Fig. 4). No increase was seen at other time points, suggesting that this increase in the osteoprogenitor population after estrogen is relatively transient. The total number of fibroblastic colony-forming units showed a similar pattern of response.

RESULTS

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DISCUSSION

Our findings demonstrate that, in intact female mice, high-dose estrogen treatment is followed by remarkably rapid changes in the size of several hematopoietic subpopulations within long bone marrow. These changes were observed as early as 1 day after treatment, whereas, in contrast, the osteogenic precursor population, as reflected by CFU-AP, did not increase until after 8 days. Consistent with the latter observation, deposition of new medullary bone as assessed by histomorphometry was not observed until 12 days after estrogen treatment, which is in line with results of our previous time course study (29). On the basis of these findings, effects of high-dose estrogen on hematopoietic bone marrow would appear to precede rather than follow those on osteogenesis. Hence, changes in hematopoiesis induced by estrogen presumably represent a direct effect on this compartment rather than a secondary phenomenon as a consequence of new bone formation reducing the space available for hematopoiesis.

One limitation of this study is that, because supraphysiological doses of estrogen were used to achieve a maximal response, it is unclear how relevant our findings are to the action of lower estrogen levels within the physiological range. However, doses of 17β-estradiol as low as 4 μg·kg⁻¹·day⁻¹ stimulate osteogenesis in mice (28), whereas physiological doses of estrogen inhibit several hematopoietic lineages as assessed by studies of the effect of ovariecotomy on hematopoiesis (6, 13, 18). Thus a similar association may exist between effects...
of physiological estrogen levels on hematopoiesis and osteogenesis to that found for high-dose estrogen. It has previously been reported that high-dose estrogen reduces the number of lymphoid and granulocytic cells within bone marrow in mice 2 wk after commencement of treatment (10). To our knowledge, no previous study has examined the effects of estrogen on hematopoietic marrow at earlier time points, as reported here. In addition, as far as we are aware, no previous study has investigated the effect of estrogen on osteogenic precursors using an ex vivo approach, as outlined in this investigation. Only a proportion of CFU-AP colonies produce mature osteoblasts capable of synthesizing bone nodules in culture, which was not assessed in the present study. Therefore, further studies are required to confirm our observations that suggest that estrogen stimulates osteoprogenitor generation in vivo. However, our results are consistent with a report that estrogen stimulates the proliferation and osteogenic differentiation of osteoblast precursors in mouse bone marrow cultures in vitro (26).

Another limitation of this study is that insufficient bone marrow cells were obtained from individual mice to permit simultaneous analysis by flow cytometry and CFU-AP assay. Therefore, the suggestion from our results that estrogen’s effects on hematopoiesis precede those on osteogenesis requires confirmation in further studies in which these responses are assessed concurrently. Although flow cytometry enabled the effect of estrogen on the size of hematopoietic subpopu-
lations to be determined, this method provided limited information with respect to the associated osteogenic response; although ALP is a marker for early osteoblast differentiation (27, 37), estrogen was found to significantly reduce the ALP-positive bone marrow fraction, presumably reflecting the fact that ALP is also expressed by hematopoietic bone marrow cells such as neutrophils (8). Hence, the ALP-positive bone marrow fraction does not mirror the bone marrow osteoprogenitor population, which in contrast increased after estrogen as assessed by counting CFU-AP colonies in ex vivo bone marrow cultures. It may also be possible to analyze estrogen-induced osteogenesis by flow cytometry by employing other markers suggested to identify the osteogenic subfraction with this technique (34, 41).

A decrease in production of myeloid and lymphoid precursors may have contributed to the decline in hematopoietic populations observed after estrogen treatment. Consistent with this possibility, estrogen administration has previously been reported to reduce the number of pre-B lymphocyte colonies in mouse bone marrow (20). Any tendency of estrogen to suppress lymphocyte precursors may be mediated by a primary interaction with stromal cells, which play a major role in regulating hematopoiesis, including B lymphopoiesis (31). In support of this hypothesis, pretreatment of stromal cells with estrogen has previously been found to suppress B lymphopoiesis in vitro (18, 35). Because stromal cells are also thought to contain the osteoprogenitor population (7, 22), it is possible that estrogen’s effects on hematopoietic bone marrow and osteogenesis...
are both mediated through an action on stromal cells. That stromal cells play a role in reciprocal regulation of hematopoiesis and osteogenesis is also suggested by studies of the senescence-accelerated mouse-P6 in which enhanced hematopoiesis and impaired osteogenesis are thought to occur as a consequence of altered stromal cell function (14).

An action on precursors may also explain our finding that estrogen expanded the erythroid population, in view of a previous report that estrogen treatment increases the number and mitotic activity of erythroid precursors in mouse spleen and bone marrow (17). On the other hand, the finding that estrogen transiently increased the number of B lymphocytes over the first 2 days may have reflected initial expansion in the population of relatively mature cells, as previously observed in mice exposed to high levels of estrogen during pregnancy (20). Estrogen’s suppressive effects on leucocytes may also have involved an effect on relatively mature cell types, such as induction of apoptosis; although we saw no morphological evidence for apoptosis, further studies are required to address this question in more detail.

Our observation that estrogen transiently increases the number of megakaryocytes within bone marrow is consistent with previous findings (17). In addition, a more sustained increase in megakaryocyte number has been reported in association with elevated levels of estrogen during pregnancy in mice (19) and after estrogen replacement therapy in postmenopausal women (2). Regulation of megakaryocyte number by estrogen in this way may be mediated directly through binding with megakaryocyte estrogen receptors (2, 38). Transgenically induced megakaryocytosis in mice has previously been reported to induce osteogenesis, possibly through increased release of megakaryocyte-derived osteogenic growth factors like platelet-derived growth factor (43). The latter is known to induce profound stimulation ofmurine fibroblastic colony-forming units (16, 42) and is itself induced by estrogen (30). Therefore, effects of estrogen on megakaryocytes represent a further potential mechanism by which hematopoietic populations might mediate estrogen-induced osteogenesis.

In summary, we have found that high-dose estrogen leads to a rapid decline in hematopoietic populations within mouse long bone marrow. Moreover, this response appears to precede estrogen’s associated stimulatory action on bone marrow osteogenic precursors. Therefore, suppression of hematopoiesis after estrogen treatment in this species does not appear to result from loss of marrow space as a consequence of estrogen’s stimulatory action on osteogenesis. Further studies are required to characterize estrogen’s reciprocal effects on hematopoiesis and osteogenesis in more detail, for example, by analyzing whether stromal cells play an important role in mediating these two responses.

This study was funded by the Arthritis Research Campaign.

REFERENCES


