Estrogen Deficiency Increases the Ability of Stromal Cells to Support Murine Osteoclastogenesis via an Interleukin-1- and Tumor Necrosis Factor-mediated Stimulation of Macrophage Colony-stimulating Factor Production*

(Received for publication, April 9, 1996, and in revised form, July 9, 1996)

Robert B. Kimble, Sunil Srivastava, F. Patrick Ross, Alicia Matayoshi, and Roberto Pacifici

From the Division of Bone and Mineral Diseases and the Department of Pathology, Washington University School of Medicine and Barnes/Jewish Hospital, St. Louis, Missouri 63110

To analyze how estrogen blocks osteoclastogenesis, we investigated the effects of ovariectomy on osteoclast (OC) formation in co-cultures of purified OC precursors and purified stromal cells (SC). OC formation was higher in co-cultures containing SC from ovariec-tomized mice than in those containing SC from sham-operated mice, thus suggesting that estrogen regulates osteoclastogenesis by targeting SC. Ovariectomy also increased the mononuclear cell secretion of interleukin (IL)-1 and tumor necrosis factor (TNF) and the SC production of macrophage colony-stimulating factor (M-CSF). Osteoclastogenesis and SC production of M-CSF were not blocked by in vitro estrogen treatment but were decreased by in vivo treatment of donor mice with either estrogen or a combination of the IL-1 inhibitor, IL-1 receptor antagonist, and the TNF inhibitor, TNF binding protein. IL-1 and TNF production were also blocked by in vivo estrogen treatment, demonstrating that the increased bone marrow levels of IL-1 and TNF characteristic of ovariectomized mice induce the formation of a SC population characterized by a high production of M-CSF and increased pro-osteoclastogenic activity. Since in co-cultures of SC and OC precursors M-CSF levels correlated with OC production (r = 0.7, p < 0.0001), the data also indicate that the pro-osteoclastogenic activity of SC is proportional to their secretion of M-CSF. The ability of estrogen to decrease SC production of M-CSF and the pro-osteoclastogenic activity of these cells by regulating IL-1 and TNF production is a previously undescribed mechanism by which estrogen down-regulates osteoclastogenesis.

Osteoclastogenesis is a complex phenomenon that is facilitated by the interaction of bone marrow stromal cells (SC) with hematopoietic OC precursors. SC contribute to osteoclastogenesis by providing a physical support for nascent OCs and by producing soluble and membrane-associated factors that stimulate the proliferation and/or the differentiation of hematopoietic OC precursors (3). Among these factors are M-CSF (3–5), interleukin (IL)-6 (6), and IL-11 (7).

M-CSF is essential for the proliferation and differentiation of OC precursors (5) and appears to play a critical role in murine osteoclastogenesis because antibodies against this cytokine completely block OC formation in bone marrow cultures (5). The relevance of M-CSF is further demonstrated by the ability of M-CSF replacement to cure osteopetrosis in op/op mice, a strain characterized by the production of defective M-CSF (3). SC production of M-CSF is induced by IL-1 and tumor necrosis factor (TNF) α and β (8, 9), cytokines produced mainly by bone marrow mononuclear cells (10, 11) and recognized for their ability to promote OC formation and bone resorption (12, 13).

Thus, since estrogen receptors are expressed in mononuclear cells (14, 15), SC (16), and OC precursors (17), estrogen could block osteoclastogenesis by regulating one or more of these cell types. For example, estrogen could block the production of IL-1 and TNF from SC of the monocyte/macrophage lineage, the SC production of pro-osteoclastogenic cytokines, or the response of SC precursors to these factors.

To determine which cells, among those involved in osteoclastogenesis, are the main targets of estrogen, we have analyzed the effects of ovariectomy and estrogen replacement on purified murine SC and OC precursors. We report that the increased bone marrow cell production of IL-1 and TNF caused by estrogen deficiency leads to the expansion of a SC population characterized by a high production of M-CSF and increased pro-osteoclastogenic activity.

MATERIALS AND METHODS

All animal procedures were approved by the Animal Care and Use Committee of the Jewish Hospital of St. Louis. Unless otherwise specified, reagents and media were from Sigma.

Study Protocol—C57Bl/6J mice (Jackson Laboratory, Bar Harbor, ME) 5 weeks of age were OVX or sham-operated by the dorsal approach under general anesthesia, as described previously (18). OVX mice were either left untreated or treated with 17β-estradiol (0.16 μg/day, the lowest dose that maintains a normal uterine weight) for 2 weeks using slow releasing subcutaneous pellets (Innovative Research of America, Toledo, OH), implanted in a nuchal subcutaneous pocket. For some experiments, OVX or sham-operated mice were treated with the IL-1 inhibitor, IL-1 receptor antagonist (IL-1ra) (25 μg/kg body weight/day), plus the TNF inhibitor, TNF binding protein (TNFbp) (1 μg/kg body weight/day) during the first 2 weeks after surgery, as described previously (18). IL-1ra binds to IL-1 receptors and competes with both IL-1α and IL-1β without detectable IL-1 agonistic effects (19). TNFbp is
a specific TNF inhibitor made of two molecules of the extracellular domain of the human type I TNF receptor linked to a molecule of polyethylene glycol. TNFbp binds with equal affinity to TNFα and TNFβ. IL-1ra and TNFbp were kindly provided by Amgen Inc. (Thousand Oaks, CA). IL-1ra was administered by implanting Alzet 2002 osmotic pumps (Alza Inc., Palo Alto, CA) in a dorsal subcutaneous pocket at the time of surgery. TNFbp was injected subcutaneously every other day.

Two weeks after surgery, mice were sacrificed, femora and tibiae were excised, and bone marrow was flushed with ice-cold α-minimal essential medium (α-MEM) as described previously (18).

### Bone Marrow Cell Cultures

At sacrifice, bone marrow was flushed, bone marrow cells collected, pelleted, resuspended in α-MEM supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Inc.) and cultured in multwell plates (Becton Dickinson Labware, Lincoln Park, NY) at a density of 2.5 x 10^6/cm². Experiments designed to assess the production of M-CSF, IL-1, or TNF, bone marrow cells were cultured for 1–15 days in absence of exogenous stimulation. For experiments designed to determine the effect of ovariectomy on OC formation, bone marrow cells were cultured for 7 days in the presence of 10 nM 1,25(OH)₂D₃. At 4 days of culture, 90% of the medium was replaced, and 1,25(OH)₂D₃ was added again at the same concentration. These culture conditions were selected because in earlier studies (18) we had determined that the OC number peaks at 7 days.

### Bone Marrow Adherent Cell Cultures

To investigate the effects of ovariectomy on the production of IL-1 and TNF from cells of the monocyte/macrophage lineage, unfractionated bone marrow cells were collected as described above and fractionated on Ficoll Hypaque to prepare mononuclear cell cultures as described (20). The bone marrow mononuclear cells were seeded 5 x 10⁶ cells/ml and cultured for 1 h to allow the adherence of mononuclear cells. The culture wells were then washed twice to remove the nonadherent cells, and the adherent cells were cultured for 72 h in the absence of exogenous stimulation.

### Nonadherent OC Precursor Preparation

For some experiments bone marrow cells were harvested as described above and cultured in a Petri dish for 24 h with 10% FBS α-MEM in 150-mm culture dishes at a density of 2.5 x 10⁶/cm². Nonadherent cells were removed from the Petri dishes and washed once with serum-free α-MEM. The washed cells were then incubated in a 0.02% Pronase, 1.5 mM 3NaEDTA solution for 15 min at 37°C. After stopping the enzyme activity by adding horse serum (1:50), the cell suspension was layered onto an ice-cold donor horse serum gradient and kept on ice for 15 min. The cells were recovered from the upper layer of the gradient and layered onto a second cold horse serum gradient. The second gradient was centrifuged, and the cell pellet was resuspended in α-MEM with 10% FBS. This cell fraction, which is known to contain early OC precursors (21), was plated at a density of 5 x 10⁶/cm² in 24-well plates to be co-cultured with purified stromal cells and/or ST2 cells or utilized for assessing M-CSF production.

### Stromal Cell Preparation

Bone marrow cells were disaggregated, centrifuged, and resuspended in α-MEM containing 10% FBS and 10% horse serum. The cells were then plated at a density of 1.0–1.3 x 10⁶ cells/cm² and cultured for 7 days. The culture plates were washed with PBS to remove nonadherent cells and treated with collagenase (1 µg/ml) and trypsin to mobilize the SC. To remove contaminating macrophages from the mobilized SC, the cells were collected and treated according to the methods of Modderman et al. (22). Briefly, the cells were collected, centrifuged, washed 2 times with Ca²⁺/Mg²⁺-free PBS and resuspended in 2 µm ATP. After 5 min of incubation, 1 µM KSCN was added, and the cells were incubated for 25 min at 37°C. This was followed by termination of permeabilization with the addition of 4 µM MgCl₂. The cells were rinsed 2 times with serum-free media, resuspended in α-MEM (10% FBS and 10% horse serum) and cultured for 24 h in a Petri dish. The SC were then trypsinized and subjected to a positive selection process using magnetic beads coupled to anti-vascular cell adhesion molecule-1 antibody M/K 1.9 (23) according to the manufacturer’s instructions (Dynal Inc., Great Neck, NY). Purified SC coupled to the magnetic beads were plated in α-MEM with 10% FBS and 10% horse serum and incubated for 24 h. During this time the beads detached from the SC and were phagocytosed by contaminating monocytic cells. The cells were then trypsinized and exposed to a magnet to remove residual phagocytic cells. The purified SC were then plated and grown to confluency.

This purification procedure yielded a population of fibroblast-like cells that were >98% nonspecific esterase-negative (Fig. 1A). Control cultures that were not subjected to the immunological purification (Fig. 1B) contained numerous nonspecific esterase-positive cells. The lack of monocyte/macrophage contamination was further demonstrated by the absence of nonspecific esterase-positive cells in SC cultured for 2 more weeks. This length of time is sufficient for early monocyte precursors to differentiate into mature cells.

### IL-1 and TNF Assay

IL-1 and TNF levels were measured in the 72-h culture medium of unstimulated adherent mononuclear cells prepared as described above. IL-1 bioactivity was measured by assessing the increment in mitogen-induced proliferation of the helper T cell D10.G4.1 (D10 cells) as described previously (18, 20). The sensitivity of this assay was 1–10 pg/ml. The nature of the assayed material was confirmed as IL-1 by demonstrating inhibition of the conditioned medium effect on the D10 cell proliferation in the presence of 50 ng/ml IL-1ra. TNF was measured by a specific double site enzyme-linked immunosorbent assay, as described previously (18), using antibodies (Pharmingen, San Diego, CA) that recognize both TNFα and TNFβ. The sensitivity of this assay was 25 pg/ml.

### M-CSF Assay

M-CSF was measured using the specific double site enzyme-linked immunosorbent assay described by Perkins and Kling (24) in the culture media of confluent SC either unstimulated or stimulated with IL-1 and TNF (10 ng/ml each) for 24–72 h and in the 9-day...
culture media of unstimulated co-cultures of SC and OC precursors. M-CSF was also measured in the culture media of unstimulated non-adherent OC precursors. This assay makes use of anti-M-CSF antibodies isolated from serum-free 5A1 and D24 hybridomas conditioned media (25), by protein G chromatography. These hybridomas were a kind gift of Dr. H. S. Lin (Washington University, St. Louis, MO). The 5A1 hybridoma produces an antibody (primary antibody) that neutralizes murine M-CSF in cultures. Detection of M-CSF was carried out using immobilized D24 antibody, with signal amplification by streptavidin-horseradish peroxidase. The lower limit of detection of this assay was 0.15 ng/mL (1600 units = 1 ng of M-CSF).

RNA Purification and Northern Blot Analysis—Total cellular RNA was isolated by the single-step acid guanidinium thiocyanate-phenol-chloroform method described by Chomczynski et al. (26), quantified by absorbance spectroscopy, and electrophoresed on 1% agarose gels in Northern buffer. The RNA was transferred onto zeta probe nylon membranes with a vacuum blotter. The membranes were prehybridized with hybridization buffer (5 x SSPE, 5 x Denhardt’s solution, 50% formamide, 0.1% SDS, 1 x background quencher (Tel-Test, Inc., Friendswood, TX)) for 2 h at 42 °C and hybridized in hybridization buffer with 32P-labeled probes for 16 h at 42 °C. The membranes were then washed with 2 x SSC, 0.1% SDS for 30 min at 42 °C and 0.2 x SSC, 0.1% SDS for 30 min at 60 °C and exposed to Kodak X-OMAT film for 2 days at −80 °C. As an M-CSF probe, we used a 4.1-kilobase pair cDNA specific for murine M-CSF (27) (American Type Culture Collection, Rockville, MD). A cDNA specific for 18 S mRNA was also used as a control. Both probes were [32P]dCTP-labeled by the random priming method (Boehringer Mannheim).

Co-cultures of ST2 Stromal Cells and OC Precursors—For some experiments ST2 stromal cells (Riken, Tukuba, Japan) were seeded (5 x 104 cells/cm2) in 24-well plates and co-cultured with nonadherent OC precursors (5 x 104 cells/cm2) for 7 days in α-MEM supplemented with 10% fetal calf serum, 10−7 M dexamethasone, and 10 nM 1,25(OH)2D3. Co-cultures of Bone Marrow SC and OC Precursors—Co-culture experiments were performed by incubating purified bone marrow SC and purified nonadherent OC precursors obtained from OVX and sham-operated mice groups. These experiments were carried out by seeding in 24-well plates purified SC (5 x 104 cells/cm2) obtained from OVX (or sham-operated) mice and nonadherent OC precursors (5 x 104 cells/cm2) obtained from sham-operated (or OVX) mice and culturing these cells for 7 days in α-MEM supplemented with 10% fetal calf serum and 10 nM 1,25(OH)2D3.

OC Characterization—At the end of the culture period, unfraccionated bone marrow cells or co-cultures of SC and OC precursors were fixed and stained for tartrate-resistant acid phosphatase using a commercial Tartrate-resistant acid phosphatase-positive cell kit (Sigma) and three or more nuclei were counted as OC-like cells. Expression of calcitonin receptors was also assessed by autoradiography using 125I-labeled salmon calcitonin (Peninsula Laboratories Inc., Belmont, CA), as described previously (18). More than 98% of the tartrate-resistant acid phosphatase-positive multinucleated cells formed in the bone marrow cultures showed specific binding of labeled calcitonin. Therefore, we regarded the tartrate-resistant acid phosphatase-positive multinucleated cells formed in the bone marrow cultures as authentic OC-like cells.

Statistical Analysis—Group mean values were compared by two-tailed Student’s t test or one-way analysis of variance as appropriate. Subsequent mean comparison tests were performed by Fisher protected least significant difference test. Simple linear regression analysis was used to determine the relationship between M-CSF levels and the OC number. The likelihood of type 2 errors was evaluated by calculating the power of unpaired two-tailed t tests (28) to detect significant differences in M-CSF levels between media from untreated cells and cells treated in vitro with 17β-estradiol.

RESULTS

Effect of Ovariectomy on the Production of Osteoclast-like Cells—In the present study we utilized unfraccionated bone marrow cell cultures (in which both SC and OC precursors originate from the same donor) to investigate the effect of ovariectomy on osteoclastogenesis. As previously reported (18), the number of bone marrow cells obtained from untreated OVX mice was higher than that derived from sham-operated mice (not shown). Additional, unfraccionated bone marrow cells obtained from OVX mice produced a 2-fold increase in the number of OC over cells from either untreated sham-operated or estrogen-treated OVX mice. This difference was observed when the results were expressed either as number of OC/106 bone marrow cells (Fig. 2A) or as OC/mouse (not shown). These results confirm previous observations by us and others (6, 18).

To investigate whether ovariectomy increases osteoclastogenesis via an effect on OC precursors, nonadherent bone marrow cells (early OC precursors) obtained from OVX and sham-operated were stimulated with 1,25(OH)2D3, ST2 stromal cells, and co-cultured with ST2 stromal cells, a cloned cell line that promotes OC formation from bone marrow cells, spleen cells, and monocytes (29). These experiments revealed (Fig. 2B) that when ST2 cells are used as a common source of SC, ovariectomy had no effect on the production of OC. The number of OC was, in fact, similar in co-cultures of ST2 cells and OC precursors from sham-operated, untreated OVX, or estrogen-treated OVX mice. The number of OC scored in these co-cultures was 5-fold higher than that found in the unfraccionated bone marrow cultures, a finding consistent with the powerful pro-osteoclastogenic effects of ST2 cells (30). Taken together, these data suggest that the presence of SC from OVX mice is required to unveil the stimulatory effect of estrogen deficiency on osteoclastogenesis.

To confirm that ovariectomy stimulates osteoclastogenesis by conditioning the ability of SC to support the proliferation and/or differentiation of OC precursors, bone marrow cells from sham-operated and OVX mice were fractionated into SC and nonadherent OC precursors as described under “Materials and Methods.” Nonadherent OC precursors from OVX (or sham-operated) mice were then cultured with SC from sham-operated (or OVX) mice in the presence of 1,25(OH)2D3 for 7 days. Fig. 3 shows that each of the co-cultures containing SC from OVX mice produced a 2-fold increase in the number of OC than co-cultures containing SC from sham-operated mice. In contrast, the source of nonadherent OC precursors did not alter the number of OCs produced by the co-cultures, as indicated by
Fig. 3. Effect of ovariectomy (mean ± S.E.) on OC formation in vitro in co-cultures of bone marrow SC and nonadherent OC precursors. Nonadherent OC precursors and bone marrow SC were purified as described under “Materials and Methods” from OVX and sham-operated mice (n = 6 per group) sacrificed 2 weeks after surgery and cultured for 7 days with 10 nM 1,25(OH)D₃. Co-cultures containing SC from OVX mice produced a larger number of OC than co-cultures containing SC from sham-operated mice. Results (mean of four experiments) were expressed as OC/10⁶ nonadherent cells. ∗, p < 0.01 compared with other groups.

The finding of a similar number of OCs in groups containing OC precursors originating from either sham-operated or OVX mice. These findings confirm that estrogen down-regulates osteoclastogenesis via an effect on SC.

Effect of Ovariectomy and Estrogen Treatment on the Stromal Cell Production of M-CSF—In order to test the hypothesis that ovariectomy and estrogen treatment regulates osteoclastogenesis by modulating the production of soluble factors that promote the proliferation and/or differentiation of OC precursors, we investigated the effect of ovariectomy and in vitro estrogen treatment on the production of M-CSF by unfractonated bone marrow cells and purified SC obtained from sham-operated and OVX mice (both untreated and estrogen-treated). Time course experiments revealed (Fig. 4A) that in the first 2 weeks of culture M-CSF levels were about 2-fold higher in conditioned media from unstimulated bone marrow cells from OVX mice than in those from either sham-operated or estrogen-treated OVX mice.

To determine the cell type responsible for this phenomenon, M-CSF levels were measured in the culture media of either purified unstimulated SC or purified nonadherent OC precursors. These experiments revealed that between day 9 (the earliest time point when a sufficient amount of SC can be purified) and day 15 of the culture, purified SC from OVX mice produced higher M-CSF levels (Fig. 4B) than SC from either sham-operated or estrogen-treated OVX mice. These findings did not change when the data were expressed as units/10⁶ cells rather than as units/ml medium or when SC were stimulated with IL-1 and TNF during the last 24 h of the culture period (not shown). Conversely, purified OC precursors from all groups secreted low levels of M-CSF (<150 units/ml), and ovariectomy did not increase the ability of these cells to secrete M-CSF (not shown).

Since the production of M-CSF is primarily regulated at the transcriptional level (31), we then examined the effect of ovariectomy on the steady state expression of M-CSF mRNA by Northern blot analysis. Fig. 5A shows that confluent murine SC stimulated with IL-1 and TNF express a transcript of about 4.5 kilobase pairs. Northern blot analysis also revealed (Fig. 5, A and B) a higher M-CSF mRNA expression in IL-1- and TNF-stimulated SC from OVX mice than in those from sham-operated mice.

We then asked whether estrogen has direct effects on the SC production of M-CSF. For these experiments, purified SC were prepared from bone marrow of sham-operated, OVX, and estrogen-treated OVX mice and cultured from harvest to confluence in phenol red-free medium and charcoal-stripped serum. The cells were then treated with 11β-estradiol (10⁻⁸ to 10⁻¹² M) for 24–72 h and stimulated with IL-1 and TNF for the last 4–24 h of the culture period. The culture medium of cells stimulated with IL-1 and TNF for 24 h was then collected and assessed for M-CSF levels by enzyme-linked immunosorbent assay. Cells stimulated with IL-1 and TNF for 4 h were harvested and analyzed for M-CSF mRNA steady state expression, as described above. These experiments revealed that under the experimental conditions described above, in vitro estrogen treatment does not decrease the stromal cell production of M-CSF (Fig. 6) and the expression of M-CSF mRNA (not shown). These data suggest, although they do not conclusively demonstrate, that estrogen does not regulate the production of M-CSF via a direct effect on SC.

The power of this analysis was >0.9 (28), suggesting that the lack of response to in vitro estrogen treatment was not due to an insufficient number of observations.

Effects of IL-1 and TNF on the in Vitro SC Production of M-CSF—Since in vivo estrogen treatment decreases SC production of M-CSF, whereas in vitro estrogen treatment does not, the possibility exists that in estrogen-replete mice SC precursors differentiate into a phenotype characterized by a lower production of M-CSF. Conversely, in estrogen-deficient mice SC precursors may differentiate into a high M-CSF producing phenotype. Since IL-1 and TNF are known to regulate the differentiation of OC precursors (9) and to induce M-CSF production (32) and since our data revealed that IL-1- and TNF-stimulated SC from OVX mice produce more M-CSF than cells from control mice, we hypothesized that the increased production of IL-1 and TNF that characterizes the bone marrow of OVX mice leads to the generation of a high M-CSF-producing SC phenotype. To test this hypothesis, we first measured IL-1 and TNF levels in the culture media of unfractonated bone marrow cells from OVX and sham-operated mice. These experiments revealed (Table 1) that 2 weeks after surgery unfractonated bone marrow cells from OVX mice produce higher (about 3-fold) levels of IL-1 and TNF than cells from either sham-operated or estrogen-treated OVX mice. An increased production of IL-1 and TNF from cells from OVX mice was also observed in cultures of adherent bone marrow mononuclear cells, thus demonstrating that estrogen regulates the production of IL-1 and TNF from cells of the monocyte/macrophage lineage.

We then analyzed the effects of in vitro treatment with IL-1 and TNF on the secretion of M-CSF from SC obtained from intact mice. These time course experiments revealed that the culture media of SC treated with IL-1 and TNF (10 ng/ml each) for at least 72 h contain higher (p < 0.05) levels of M-CSF (3402 ± 65 units/ml) than media from unstimulated SC (2440 ± 110 units/ml). Conversely, treatment with IL-1 and TNF for 24–48 h had no effect on M-CSF production. Since long term treatment of rapidly proliferating SC was required to up-regulate M-CSF production, the data appeared consistent with the hypothesis that IL-1 and TNF induce the formation of a high M-CSF-producing SC population.

To further investigate this matter, we treated an additional group of OVX and control mice with IL-1ra and TNFp for the first 2 weeks after surgery. At the end of the treatment, bone marrow cells were harvested and SC purified and cultured until confluence. The culture medium was then harvested and assayed for M-CSF. These experiments revealed (Fig. 7) that SC from OVX mice pretreated with either IL-1ra and TNFp or estrogen produced amounts of M-CSF lower than those of SC.
from untreated OVX mice and similar to those of SC from either sham-operated or estrogen-treated OVX mice. M-CSF levels were not measured in the SC conditioned medium earlier than day 9 because insufficient amounts of SC can be purified at earlier time points. *, p < 0.01 compared with the other groups. Purified OC precursors produced negligible amounts (≤150 units/ml) of M-CSF at all times. Ovariectomy and estrogen replacement had no effects on the production of M-CSF from purified OC precursors.

We then asked whether exposure to high levels of IL-1 and TNF during the bone marrow maturation is necessary for increasing the pro-osteoclastogenic activity of SC. To this aim, SC and OC precursors were purified from the bone marrow harvested from mice treated in vivo with IL-1ra and TNFbp for the first 2 weeks after surgery. Purified cells were co-cultured for 7 days with 1,25(OH)2D3 to induce the OC formation. Moreover, since M-CSF exerts its effects early in OC differentiation (3), to further investigate the role of M-CSF in the mechanism by which SC promote osteoclastogenesis, the amount of M-CSF in the media of each co-culture group was measured at day 3. Fig. 8 shows that co-cultures of SC from OVX mice pretreated in vivo with either estrogen or IL-1ra and TNFbp and OC precursor from untreated OVX mice produced fewer OC and had lower levels of M-CSF in the culture medium than co-cultures of SC from untreated OVX mice and OC precursors from untreated OVX mice. Both treatments decreased OC production to amounts similar to those generated from co-cultures of cells obtained from sham-operated mice. OC production and M-CSF levels were also partially decreased by pretreatment of OC precursors with IL-1ra and TNFbp. In contrast, pretreatment of OC precursors with estrogen had no effect.

Fig. 9 shows that M-CSF levels, measured in the culture media of co-cultures of SC and OC precursors at day 3, were significantly correlated (r = 0.70, p < 0.0001) to the number of OCs produced by each group. Taken together, these data demonstrate that the functional block of IL-1 and TNF during bone marrow maturation leads to the generation of a SC population characterized by a low pro-osteoclastogenic activity, which results from a lower production of M-CSF.

**DISCUSSION**

This study was designed to investigate which of the cells involved in murine osteoclastogenesis are influenced by estrogen. We found that in vivo estrogen treatment leads to the generation of a SC population characterized by low pro-osteoclastogenic activity and low M-CSF production (Fig. 10). We also determined that these effects are indirect, since they were not induced by in vitro estrogen treatment but were prevented by the functional block of IL-1 and TNF.

To determine the cellular targets of estrogen in the bone marrow, we analyzed the formation of OC in co-cultures of purified SC and early OC precursors (nonadherent bone marrow cells). Although it is now recognized that bone marrow cells harvested from OVX mice during the first 4 weeks from surgery produce more OC than cells from sham-operated mice (6, 18, 20), this study was designed to investigate which of the cells involved in murine osteoclastogenesis are influenced by estrogen.
33), in this study bone marrow cells were collected only at 2 weeks. This design was selected because published evidence demonstrating that sera from mice treated with IL-1ra and TNFbp neutralize IL-1 and TNF is limited to 2-week-long treatments (18). Similarly, SC and OC precursors were co-cultured for 7 days, because previous time course experiments had revealed that the number of OC scored in cultures from all groups peaks at 7 days (18).

Our findings demonstrate that bone marrow cells from OVX mice produce larger amounts of M-CSF than cells from sham-operated mice when cultured for up to 2 weeks. Although we were unable to measure M-CSF levels in freshly isolated SC due to insufficient cell number, the data obtained by culturing SC for 9 and 15 days demonstrate that SC are the source of the difference in M-CSF levels between OVX and estrogen-replete mice.

![Figure 6](image-url)

**FIG. 6.** Effect of *in vitro* estrogen treatment (mean ± S.E.) on the production of M-CSF from purified stimulated SC. Two weeks after OVX or sham operation, SC were isolated and cultured until confluence in phenol red-free medium and charcoal-stripped serum. At confluence, cells were treated with 17β-estradiol (10⁻² to 10⁻¹² M) for 24 h (A), 48 h (B), and 72 h (C) and stimulated with IL-1 and TNF (10 ng/ml each) for the last 24 h of the culture period. The culture medium was then collected and assessed for M-CSF levels by enzyme-linked immunosorbent assay. Similar results were obtained by incubating in experiments conducted with unstimulated cells (not shown).

**FIG. 7.** Effect (mean ± S.E.) of *in vivo* treatment with IL-1ra and TNFbp on the *in vitro* production of M-CSF from unstimulated SC. OVX and sham-operated mice were treated with IL-1ra and TNFbp 17β-estradiol as described under “Materials and Methods” for the first 2 weeks after surgery. At the end of the treatment, bone marrow cells were harvested, and SC was purified and cultured until confluency as described above. The culture medium was then harvested and assayed for M-CSF. Similar results were obtained using SC stimulated with IL-1 and TNF during the last 24 h of the culture period (not shown).

| Table I | Effect of ovariectomy and sham operation on the secretion of IL-1 bioactivity and TNF (mean ± S.E.) from unfractionated and adherent mononuclear bone marrow cells |
|---------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|          | Untreated sham-operated (n = 6)                                                                                                                                         | Untreated OVX (n = 6)                                                                 | Estrogen-treated OVX (n = 6)                                                                 |
| BM cells | **IL-1** (units/ml)                                                                                                 | **18.5 ± 0.7**                                                                                  | **4.6 ± 0.6**                                                                                  |
|          | **16.5 ± 0.9**                                                                                                    | **141.0 ± 18.3**                                                                                | **59.2 ± 23.5**                                                                                |
|          | **1.0 ± 0.6**                                                                                                     | **21.5 ± 5.9**                                                                                 | **18.7 ± 1.3**                                                                                 |
| Adherent BM cells | **IL-1** (units/ml)                                                                                               | **53.4 ± 11.0**                                                                                | **49.6 ± 18.7**                                                                                |
|          | **163.0 ± 21.5**                                                                                                  | **141.0 ± 18.3**                                                                                | **59.2 ± 23.5**                                                                                |
|          | **10.8 ± 3.8**                                                                                                    | **18.3 ± 3.8**                                                                                 | **13.5 ± 4.5**                                                                                 |
|          | **10.8 ± 3.8**                                                                                                    | **18.3 ± 3.8**                                                                                 | **13.5 ± 4.5**                                                                                 |

* p < 0.05 compared with the corresponding group of untreated sham-operated and estrogen-treated OVX mice.
Moreover, since it has been demonstrated that the capacity of uncommitted SC precursors to differentiate into defined phenotypes depends upon the estrogen status of the donor mouse (34), the opposite outcome of the in vivo and in vitro studies suggests that changes in the bone microenvironment induced by ovariectomy led to the selection and expansion of a SC population characterized by production of high levels of M-CSF. That this accounts, at least in part, for a high pro-osteoclastogenic activity of these cells is suggested by the finding of a direct relationship between M-CSF levels and OC production in all of the conditions examined in this study. In agreement with this hypothesis are previous reports from our laboratory and the results of the current study. These studies not only demonstrate that ovariectomy increases the mononuclear cell production of IL-1 and TNF, but they have shown that in OVX animals IL-1ra and TNFbp block bone loss, bone resorption (both in vivo and in vitro), OC formation (18, 20, 35), and the pro-osteoclastogenic activity of SC. Since IL-1ra and TNFbp have no effects in estrogen-replete animals (35), taken together these observations demonstrate that increased production of IL-1 and TNF from bone marrow mononuclear cells is one of the mechanisms by which estrogen deficiency affects SC differentiation and modulates osteoclastogenesis.

The current study also demonstrates that the mechanism by which IL-1 and TNF promote osteoclastogenesis in estrogen-deficient mice is by increasing the SC production of soluble M-CSF. Whether the production of membrane-bound M-CSF is also regulated by estrogen via IL-1 and TNF remains to be determined. Moreover, our findings do not exclude the possibility that estrogen may have additional effects on SC that contribute to the inhibitory effect of the hormone on osteoclastogenesis. For example, estrogen could down-regulate the SC production of other soluble factors known to regulate OC for-
mation, such as IL-6 and IL-11, or decrease the SC response to pro-osteoclastogenic factors.

Although the critical role of M-CSF for both the proliferation and differentiation of OC precursors has been long recognized (3,5), an effect of ovariectomy on the production of this factor has not been previously reported, presumably because in most studies the regulation of M-CSF production has been investigated using cloned SC lines rather than primary cultures (3). Our data demonstrate that both the IL-1 and TNF-induced expression of M-CSF mRNA and the secretion of M-CSF are down-regulated by in vitro but not in vivo estrogen treatment. It is unlikely that the inability of in vitro estrogen treatment to decrease M-CSF production is due the experimental conditions selected for this study, because power analysis demonstrated a low probability of a type 2 error. Moreover, not only were SC isolated and cultured in phenol red-free medium supplemented with charcoal-stripped medium, but both unstimulated and IL-1 and TNF-stimulated SC were incubated with estrogen for a length of time that has been shown to inhibit the secretion of other pro-osteoclastogenic factors (36). These findings are in keeping with both the lack of typical estrogen-responsive elements in the murine M-CSF promoter (37) and the ability of in vitro, but not in vivo, estrogen treatment to increase the expression of the ubiquitous transcription factor Egr-1 (also known as Zif 268 or NGF1A) in estrogen-responsive cells (38).

The finding that OC production is not affected by the estrogen status of the OC precursor donor suggests, although it does not conclusively demonstrate, that estrogen does not regulate the proliferation and/or the differentiation of murine OC precursors with a direct mechanism. This hypothesis is further supported by the demonstration that ovariectomy had no effect on the proliferation and/or the differentiation of murine OC precursors in the presence of high levels of IL-1 and TNF.

In conclusion, this study demonstrates that differentiation of SC precursors in the presence of high levels of IL-1 and TNF (characteristic of the bone marrow of OVX mice) leads to the generation of SC characterized by a high production of M-CSF and increased pro-osteoclastogenic activity. The ability of estrogen to modulate the SC production of M-CSF via regulation of IL-1 and TNF production is a previously undescribed mechanism by which estrogen down-regulates osteoclastogenesis.

REFERENCES

1. Manolagas, S. C., and Jilka, R. L. (1995) N. Engl. J. Med. 332, 305–311.
2. Horwitz, M. (1983) Science 260, 626–627.
3. Suda, T., Takahashi, N., and Martin, T. J. (1992) Endocr. Rev. 13, 66–80.
4. Macdonald, B. R., Mundy, G. R., Clark, S., Wang, E. A., Kuehl, T. J., Stanley, F. R., and Roodman, G. D. (1986) J. Bone Miner. Res. 1, 237–252.
5. Tanaka, S., Takahashi, N., Udagawa, N., Tamura, T., Akatsuka, T., Stanley, F. R., Kurakawa, T., and Suda, T. (1993) J. Clin. Invest. 91, 257–263.
6. Jilka, R. L., Huang, G., Giraud, T., and Winkhaus, D. C., Abrams, J. S., Boyce, B., Brommeyer, H., and Manolagas, S. C. (1992) Science 257, 88–91.
7. Giraud, G., Passeri, G., Jilka, R. L., and Manolagas, S. C. (1994) J. Clin. Invest. 93, 1516–1524.
8. Drexler, T. M., Allen, T. D., and Laity, L. G. (1977) J. Cell Physiol. 91, 335–334.
9. Drexler, T. M., Coutinho, L. H., Spooner, E., Heyworth, C. M., Daniel, C. P., Schito, R., Chang, J., and Allen, T. D. (1990) in Molecular Control of Haemopoiesis (Bock, G., and Marsh, J., eds) pp. 86–95, John Wiley & Sons Ltd., West Sussex, Unit. Kingdom.
10. Dinarello, C. A. (1991) Blood 77, 1627–1652.
11. Chaplin, D. D., and Hogquist, K. (1992) in Tumor Necrosis Factors: The Molecules and Their Emerging Role in Medicine (Beutler, B., ed) pp. 197–220, Raven Press, New York.
12. Pfeilschifter, J., Chen, C., Bird, A., Mundy, G. R., and Rodman, G. D. (1989) Eur. J. Endocrinol. 129, 113–118.
13. van den Buijzen, J. M., Most, W., and van der Ploos, L., de Groot, H., Papapoulos, S., and Lowik, C. (1991) Endocrinology 129, 1596–1604.
14. Weusten, J. L., Blankenstein, M. A., Gnei-Melting, F. H., Schuurman, H. J., Kater, L., and Thijssen, J. H. (1991) Exp. Hematol. 19, 409–414.
15. Gulshan, S., McCruden, A. B., and Stimson, W. H. (1990) Scan. J. Immunol. 31, 691–697.
16. Bellido, T., Giraud, G., Passeri, G., Yu, X. P., Mocjariza, A., Jilka, R. L., Wang, E. A., and Manolagas, S. C. (1990) Endocrinology 124, 553–562.
17. Fiorelli, G., Furi, P., Filotti, M., Tamini, A., Benvennuti, S., Seno, M., Bernabei, P., and Brandi, M. L. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 2672–2676.
18. Kizana, R., Kantine, R. B., Vannice, J. L., Kung, V. T., and Pacifici, R. (1994) J. Clin. Invest. 93, 2397–2406.
19. Aren, W. P. (1991) J. Clin. Invest. 88, 1445–1451.
20. Kizana, R., Vannice, J. L., Bloedow, D. C., Thamm, R. C., Hopfer, W., Kung, V., Brownfield, C., and Pacifici, R. (1994) J. Clin. Invest. 93, 1959–1967.
21. Clohisy, D. R., Bar-Shavit, Z., Chappel, J. C., and Teitelbaum, S. L. (1987) J. Biol. Chem. 262, 15922–15929.
22. Moulden, W. F. Vrijheid-Lammers, T., Lowik, C. W. G. M., Nijweide, P. J. Exp. Hematol. 22, 194–201.
23. Miyake, K., Medina, K., Ishihara, K., Kitamoto, M., Auerbach, R., and Kincaide, P. J. (1991) J. Cell Biol. 114, 565–575.
24. Perkins, S. L., and Kling, S. J. (1995) Am. J. Physiol. 32, E1024–E1030.
25. Lokeshwar, B. L., and Lin, H. S. (1988) J. Immunol. 141, 483–488.
26. Chomczynski, P., and Sacchi, N. (1987) Ana. Biochem. 162, 156–159.
27. Ladner, M. B., Martin, G. A., Noble, J. A., Wittman, V. P., and Brandi, M. L. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 6762–6767.
28. Kishimoto, T., and Suda, T. (1995) J. Clin. Invest. 95, 829–829.
29. Udagawa, N., Takahashi, N., Akatsuka, T., Tanaka, H., Sasaki, T., Nishihara, T., Koga, T., Martin, T. J., and Suda, T. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 7960–7964.
30. Harrington, M. A., Edenberg, H. J., Saxman, S., Pedigo, L. M., Daub, R., and Brommeyer, H. E. (1991) Gene (Amst.) 102, 165–170.
31. Suva, L. J., Harm, S. C., Gardner, R. M., and Thiade, M. A. (1991) Mol. Endocrinol. 5, 829–835.
32. Harrington, M. A., Konieczek, B., Song, A., Xia, X., Frederik, W. J., and Roodman, G. D. (1993) J. Biol. Chem. 268, 21271–21275.
33. Ackerman, S. M., Menden, A. G., Wang, Q., Liberman, C., and Yeung, C. Y. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 7525–7527.
34. Oursler, M. J., Osboly, P., Pyfferen, J., Rigs, B. L., and Speirsberg, T. C. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 6613–6617.
35. Oursler, M. J., Pederson, L., Pyfferen, J., Osboly, P., Fitzpatrick, L., and Speirsberg, T. C. (1993) Endocrinology 122, 1373–1380.
36. Hart, D. M., Forrest, C., and Baird, C. (1993) J. NIH Res. 5, 66–74.
37. Guilbert, L. J., and Stanley, R. E. (1986) J. Biol. Chem. 261, 4024–4032.