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ARTICLES

Ovariectomy-Induced Bone Loss Occurs Independently of B Cells

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Abstract Estrogen withdrawal is associated with a significant expansion in B cell precursor and mature B cell populations. However, despite significant circumstantial evidence the role of B lineage cells in ovariectomy-induced bone loss in vivo is unclear. In vitro studies have demonstrated that mature B cells have the potential to both positively and negatively impact osteoclastogenesis by virtue of their capacity to secrete pro-osteoclastogenic cytokines including receptor activator of NFκB ligand (RANKL), as well as anti-osteoclastogenic cytokines such as osteoprotegerin (OPG) and transforming growth factor beta (TGFβ). Although several studies have suggested that expansion of the B lineage following ovariectomy may play a key role in the etiology of ovariectomy-induced bone loss, in vivo studies to directly test this notion have yet to be conducted. In this study, we performed ovariectomy on μMT(−/−) mice which are specifically deficient in mature B cells. Analysis of bone mineral density (BMD) by dual-energy X-ray absorptiometry (DXA) and micro-computed tomography (CT) demonstrate that mature B cell-deficient mice undergo an identical loss of bone mass relative to wild-type (WT) control mice. Our data demonstrate that mature B cells are not central mediators of ovariectomy-induced bone loss in vivo. J. Cell. Biochem. 100: 1370–1375, 2007.

Key words: B cell; ovariectomy; osteoporosis; bone

Bone forming cells, the osteoblasts (OBs), and bone resorbing cells, the osteoclasts (OCs) are responsible for the maintenance of bone homeostasis. The physiological renewal of OCs is driven by the exposure of OC precursors bearing receptor activator of NFκB (RANK) to the key osteoclastogenic cytokine RANK ligand (RANKL), in the presence of permissive concentrations of macrophage colony simulating factor (M-CSF). OC formation is negatively regulated by osteoprotegerin (OPG), a soluble decoy receptor of RANKL and a potent physiological inhibitor of osteoclastogenesis [Teitelbaum, 2000; Khosla, 2001].

Activated lymphocytes have the capacity to secrete numerous pro- and anti-osteoclastogenic cytokines that can disrupt basal osteoclastogenesis. T cells are now well established to play key roles in promoting accelerated bone turnover that leads to bone destruction in animal models of postmenopausal osteoporosis [Cenci et al., 2000, 2003; Roggia et al., 2001; Weitzmann et al., 2002; Toraldo et al., 2003; Gao et al., 2004; Ryan et al., 2005] and in inflammation conditions such as rheumatoid arthritis [Kong et al., 1999]. By contrast the role of B lineage cells, if any, in stimulated bone turnover is poorly understood. While the total number of bone marrow myeloid cells and granulocytes does not change appreciably following ovariectomy [Masuzawa et al., 1994], estrogen deficiency potently and selectively increased the B cell precursor population [Masuzawa et al., 1994], a process that is reversed by administration of estrogen [Masuzawa et al., 1994] and raloxifene [Onoe et al., 2000; Erlandsson et al.,

Abbreviations used: BMD, bone mineral density; DXA, dual-energy X-ray absorptiometry; E, estrogen; OC, osteoclast; OB, osteoblast; OPG, osteoprotegerin; ovx, ovariectomy/ovariectomized; RANK, receptor activator of NFκB; RANKL, receptor activator of NFκB ligand; BV/TV, trabecular bone volume per tissue volume.

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2002]. Administration of estrogen to male mice similarly suppresses the number of $B220^+$ cells, a specific marker of the B cell lineage, in the bone marrow by a mechanism involving estrogen receptor alpha [Erlandsson et al., 2003]. This increase in B lymphopoiesis ultimately translates into an elevation in mature bone marrow B cells [Garcia-Perez et al., 2006]. Furthermore, ovariectomy fails to augment bone marrow B lymphopoiesis concurrent with a failure to stimulate bone resorption in granulocyte colony-stimulating factor (G-CSF) transgenic mice [Oda et al., 2005], while the lymphopoietic cytokine IL-7 when administered to mice in vivo, induces B lymphopoiesis and osteoclastic bone destruction mimicking the osteoporosis associated with estrogen deficiency [Miyaura et al., 1997]. Such studies have led to the suggestion that stimulated B lymphopoiesis is a key player in ovariectomy-induced bone resorption [Onoe et al., 2000].

Mature human B cells have been reported to possess the capacity to secrete the anti-osteoclastogenic factor OPG [Yun et al., 1998]. In addition, we have previously reported that mature peripheral blood B cells inhibit OC formation in an in vitro model of human osteoclastogenesis by a mechanism that involved, in part, TGFβ [Weitzmann et al., 2000a], a cytokine that induces apoptosis of OCs [Hughes et al., 1996; Weitzmann et al., 2000a], and is known to stimulate OPG production in OBs [Thirunavukkarasu et al., 2001]. Furthermore, depletion of B cells is reported to aggravate bone loss in vivo in an animal model of periodontitis, suggesting that B cells may act to limit bone resorption in some pathological models [Klausen et al., 1989].

In contrast, other in vitro studies have reported that both mature B cells [Han et al., 2006] and B cell precursors [Kanematsu et al., 2000] possess the capacity to secrete RANKL when appropriately stimulated. Furthermore, studies of multiple myeloma cells and cell lines have indicated the potential of these terminally differentiated plasma cells to stimulate osteoclastic bone destruction, either via direct expression of RANKL [Choi et al., 2001; Manabe et al., 2001; Heider et al., 2003] or as an indirect consequence of IL-7 secretion [Giuliani et al., 2002, 2005] which is known to stimulate bone resorption in vivo [Miyaura et al., 1997; Weitzmann et al., 2000b, 2002; Toraldo et al., 2003].

Interestingly, pluripotent $B220^+$ B cell precursors have also been demonstrated to possess the capacity to differentiate directly into bone resorbing OCs, in vitro, when exposed to M-CSF and RANKL [Sato et al., 2001; Lee et al., 2003; Toraldo et al., 2003; Blin-Wakkach et al., 2004].

Although several studies have suggested that expansion of the B lineage following ovariectomy may play a key role in the etiology of estrogen deficiency-induced bone loss, the role of mature B cells in this process remains unknown. In this study, we performed ovariectomy on μMT(−/−) mice which are specifically deficient in mature B cells [Kitamura et al., 1991]. Analysis of bone mineral density (BMD) and bone volume, demonstrate that μMT(−/−) mice undergo ovariectomy-induced bone loss in a manner indistinguishable from that of wild-type (WT) mice. Our data show that mature B cells are not central mediators of ovariectomy-induced bone loss in vivo.

MATERIALS AND METHODS

All animal procedures were approved by the IACUC of Emory University. All reagents were purchased from the Sigma Chemical Corporation (St. Louis, MO), unless otherwise indicated.

Mice

B cell-deficient μMT(−/−) mice (strain: B6.129S2-Igh-6^{tm1Cgn}/J) and genetically matched C57BL6/J WT mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed under specific pathogen-free conditions, with autoclaved water and γ-irradiated food.

Ovariectomy

At 16 weeks of age, B cell-deficient mice and WT control mice were either sham-operated, ovariectomized (ovx), or ovariectomized with estrogen replacement (ovx + E) using slow release (0.16 mg/kg/d) 17β estradiol pellets (Innovative Research of America, Sarasota, Florida) as previously described [Kitazawa et al., 1994].

Bone Mineral Density (BMD)

In vivo BMD measurements of total body, lumbar spine (spine), and femurs (average of left and right femurs from each mouse) were made by dual-energy X-ray absorptiometry (DXA) using a PIXImus2 bone densitometer

(GE Medical Systems) as previously described [Toraldo et al., 2003]. For each mouse BMD at 4 weeks was normalized to its baseline value and the data expressed as a percentage change from baseline, averaged for each group.

Micro-Computed Tomography (μCT)

One representative sample from each WT and μMT(−/−) KO group (sham mice, ovx mice, and ovariectomized mice receiving estrogen replacement) was selected further analysis by μCT. Three-dimensional (3D) core images from whole bones were reconstructed from individual μCT slices using a μCT 40 scanner, (Scanco Medical, Bassersdorf, Switzerland) as previously described [Gao et al., 2004]. Briefly, after careful dissection of muscle tissue, the right distal femur was fixed in 10% neutral buffered formalin overnight and stored in 70% ethanol at 4°C until analysis. μCT analysis was performed by an operator blinded as to the nature of the specimens. Bones were scanned at a resolution of 12 μm. For each sample 100 slices were taken at the identical position covering a total area of 1,200 μm proximal to the distal metaphyses. Static trabecular measurements of trabecular bone volume per tissue volume (BV/TV) were made using a cylindrical "core" sample that excludes cortical bone, with contouring for all subsequent slices.

Statistical Analysis

Statistical significance was determined using GraphPad InStat version 3 for Windows XP (GraphPad Software, San Diego, CA). Multiple comparisons were performed by one-way ANOVA with Tukey–Kramer post-test. $P \leq 0.05$ was considered statistically significant.

RESULTS

WT and μMT(−/−) B-Cell KO Mice Undergo an Identical Loss of Bone Mineral Density Following Ovariectomy

To investigate a possible role for mature B cells in the bone loss associated with estrogen deficiency, we performed ovariectomy on 16-week-old μMT(−/−) B cell KO mice, and genetically matched C57BL6 WT control mice. BMD in WT and μMT(−/−) mice was quantitated by DXA, a technique which provides an integrated measurement of both cortical and trabecular bone, at baseline and 4 weeks following surgery. Interestingly, μMT(−/−) mice displayed a decreased baseline relative to WT mice (-4.5 ± 0.1 at total body, -6.1 ± 0.1 at lumbar spine, and -2.9 ± 0.4 in the femurs). The data revealed (Fig. 1) that when normalized to baseline BMD values, ovariectomized WT and μMT(−/−) mice undergo a significant, and quantitatively identical loss of BMD at multiple anatomical positions including total body (Fig. 1A), lumbar spine (Fig. 1B), and femur (Fig. 1C).

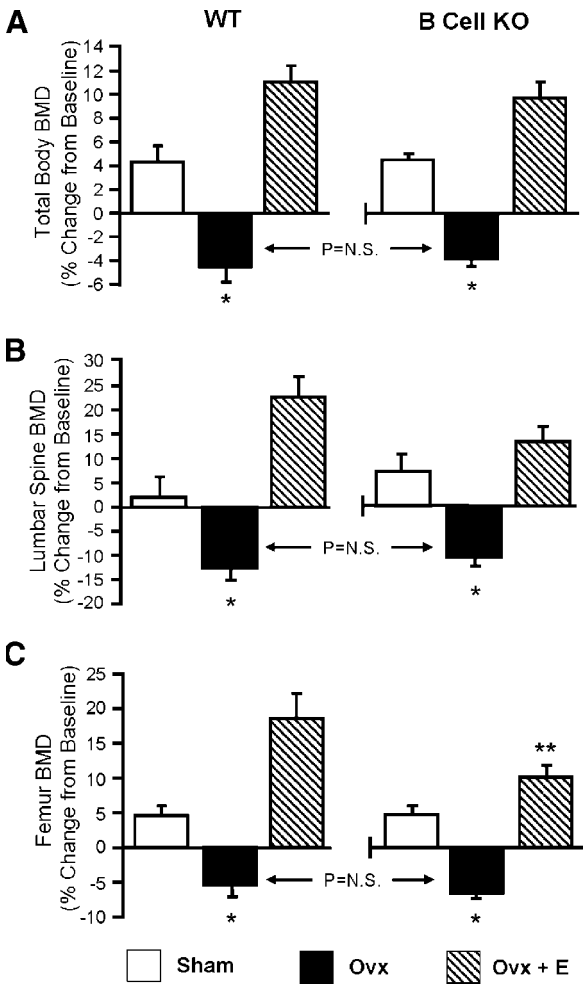

Fig. 1. B cell-deficient mice undergo significant loss of BMD following ovariectomy. Sham operation (sham), ovariectomy (ovx), or ovariectomy with estrogen replacement (ovx + E) was performed on 16-week-old μMT(−/−) B cell KO mice, and age and genetically matched C57BL6 WT control mice. BMD was quantitated at baseline and at 4 weeks following surgery in all mice at total body (**A**), lumbar spine (**B**), and femurs (**C**). Data presented as percentage change from baseline for each mouse (average ± SEM). n = 7 mice/group for WT sham and ovx and n = 6 mice/group for WT ovx + E, n = 8 mice/group for all μMT(−/−) groups. *$P < 0.001$ with respect to Sham. **$P < 0.05$ with respect to WT ovx + E. P = N.S. (not significant) relative to WT ovx (ANOVA).

B-Cell-Deficient Mice Undergo Significant Trabecular Bone Loss Following Ovariectomy

We further evaluated the femoral trabecular bone compartments of one representative WT and μMT(−/−) sample from each group of sham-operated, ovx, and ovx + E by μCT. Cross-sectional 12 μm reconstruction are shown for each group, and reveal significant loss of trabecular bone volume in both WT and B cell KO mice following ovariectomy (Fig. 2). The BV/TV computed from the images is shown below each panel. The images and BV/TVs confirmed a decreased basal bone volume in μMT(−/−) relative to WT mice.

DISCUSSION

Ovariectomy leads to a significant expansion in B cells [Garcia-Perez et al., 2006]. However, whether or not B cells play any direct role in ovariectomy-induced bone loss in vivo remains to be established. Our study demonstrates that the absence of mature B cells has no identifiable impact on ovariectomy-induced bone loss in mice, suggesting that mature B cells are not essential to the mechanism by which estrogen deficiency drives bone destruction. Furthermore, the anabolic response of estrogen administration also occurred independently of the presence or absence of B cells, as observed by both DXA and μCT.

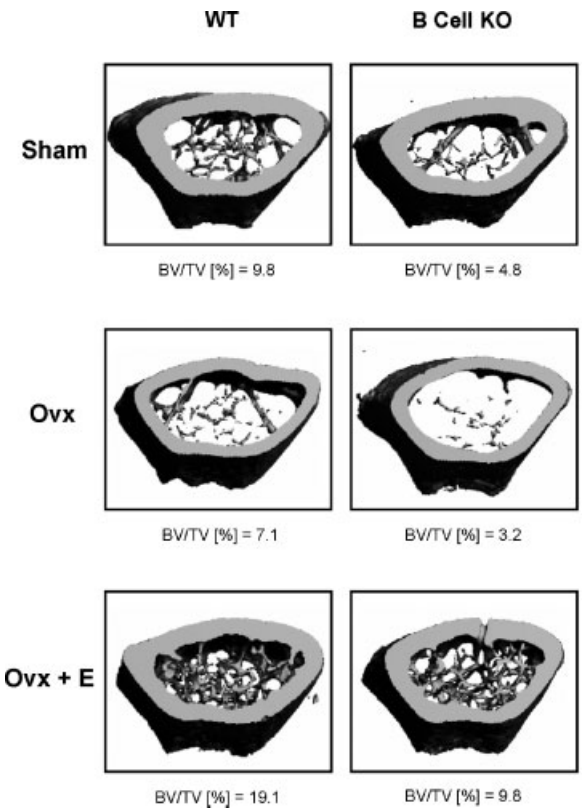

Fig. 2. B cell-deficient mice undergo significant trabecular bone loss following ovariectomy. The trabecular bone compartments of Sham-operated (sham), ovariectomized (ovx), or ovariectomized with estrogen replacement (ovx + E), WT and μMT(−/−) mice were analyzed cross-sectionally by μCT at 4 weeks following surgery. Representative 12 μm cross-sections of the distal femoral metaphyses are shown for one representative bone from each group of WT and μMT(−/−) mice. BV/TV is shown below for each sample.

The class M (IgM) antibody is the first to be expressed on the membrane of developing B cells. In the precursors of B-lymphocytes, the pre-B cells produce the heavy chain of IgM (μ chain), but not light chains. Expression of this μ chain is necessary for pre-B cell differentiation, in particular the rearrangement of the light-chain genes. Consequently disruption of this chain due to a targeted deletion of the gene encoding the μ-chain constant region leads to homozygous animals (μMT(−/−)) in which mature B cells are absent, their development already being arrested at the stage of pre-B cell maturation [Kitamura et al., 1991]. However, these mice are still replete in immature B cell populations. Early $B220^+$ IgM^- B cells have been found to be pluripotent and are capable of differentiation into OCs in vitro, in the presence of M-CSF and RANKL [Sato et al., 2001; Lee et al., 2003; Toraldo et al., 2003; Blin-Wakkach et al., 2004]. These cells are also capable of secreting RANKL [Kanematsu et al., 2000]. Consequently, our data cannot rule out a possible role for early B cell precursors, in ovariectomy-induced bone loss. Interestingly, IL-7, a potent lymphopoietic cytokine has been shown to mimic the bone loss and expansion of $B220^+$ cells observed following ovariectomy [Miyaura et al., 1997]. As IL-7 is upregulated in the BM, and at other sites, by estrogen deficiency [Weitzmann et al., 2002; Ryan et al., 2005; Lindberg et al., 2006] this cytokine is likely responsible for the expansion of $B220^+$ cells following ovariectomy. Furthermore, we have reported that IL-7 ablation using neutralizing antibodies prevents ovariectomy-induced bone loss [Weitzmann et al., 2002; Ryan et al., 2005]. We also reported that administration of IL-7 in WT mice led to pronounced bone loss, but that T cell-deficient nude mice failed to undergo bone destruction unless T cells were transplanted back. In this study, IL-7 induced a

significant expansion in $B220^+$ precursor populations in both WT and nude mice [Toraldo et al., 2003]. This suggested that expansion of $B220^+$ cells, is either not obligatory for ovariectomy-induced bone loss, or that T cell-derived cytokines are additionally necessary to promote $B220^+$ cell differentiation into OCs. Further studies will be required to assess the role of B cell precursors in estrogen deficiency-induced bone loss in vivo.

Interestingly, our data revealed that basal BMD and bone volume are significantly diminished in B cell KO mice relative to genetically matched WT controls at identical age. Despite beginning at a reduced basal BMD, B cell KO mice lost an identical amount of bone following ovariectomy, as compared to WT mice. The reason for the reduced baseline BMD is presently under investigation, however, the responsible mechanism involved appears to operate independently of ovariectomy, as the magnitude of bone loss was neither exacerbated nor reduced in B cell KO mice relative to WT mice.

Estrogen replacement in ovx mice not only prevented bone loss in both WT and B cell KO mice, but induced an expected anabolic response leading to elevated bone mass relative to the respective sham-operated mice. However, the magnitude of the anabolic response to 17β-estradiol was more pronounced in WT mice than B cell KO mice. This difference was most evident at the femurs where the estrogen driven increase in BMD in B cell KO mice was significantly decreased ($P < 0.05$, ANOVA), achieving only ~50% of the stimulation observed in WT mice. Femoral μCT analysis likewise ratified this difference in response. The reason for this decreased accumulation of bone mass in response to estrogen is not presently known but suggests the intriguing possibility that the anabolic effect of estrogen is mediated in part, through B cells. We have previously reported that B cells have the capacity to secrete large amounts of TGFβ [Weitzmann et al., 2000a], a cytokine whose concentration is stimulated by estrogen [Robinson et al., 1996]. Among its many effects TGFβ is thought to play an essential role in directing the initial commitment of stromal cell differentiation towards the OB phenotype [Janssens et al., 2005]. A lack of B cell-derived TGFβ production following estrogen administration in the B cell KO mouse may have resulted in a failure of estrogen to maximally drive new bone formation.

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