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Hormonal Changes Affect the Bone and Bone Marrow Cells in a Rat Model

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Abstract In this study, we used a rat model to investigate the effects of gonad hormones and replacement therapy on bone structure and the immune system. In the first phase of the study, 3- and 11-month-old F344 rats underwent ovariectomy (OVX) or were sham operated. Three months later, severe osteopenia was histologically observed in OVX rats of both age groups. The changes in the bone marrow structure of OVX rats included deterioration of cancellous bone that was associated with a remarkable increase of adipocyte cells. Furthermore, differential analyses for the expression of cell surface antigens by lymph-myeloid cells was studied using flow cytometry (FACS). The number of myeloid cells expressing ED-9$^+$ or CD-44$^+$ was similar in both age groups, and unaffected by OVX. However, an augmentation of T-lymphoid cells expressing CD4$^+$, CD5$^+$, or both, were observed with age, as well as after OVX. In the second phase of the study, 11-month-old rats were divided into five experimental groups: sham-operated, OVX, and OVX treated with sustained-release pellets of 17β-estradiol (OVX-E), progesterone (OVX-P), or both (OVX-E/P). Hormone replacement therapy maintained low physiological levels, and rats were tested 12 weeks after treatment initiation. Administration of 17β-E, with or without the addition of progesterone, prevented the rise of T lymphoid cells observed in OVX rats, whereas progesterone alone had no effect. In agreement with findings from the first phase, neither OVX nor replacement therapy affected the myeloid cells expression of ED-9 or CD-44. In summary, the cellular changes in the bone marrow of OVX rats were associated with an increase in adipocytes that was correlated with bone atrophy. An augmentation of T-lymphopoiesis was noted with increase in age or after OVX. This increase was reversed to baseline levels by 17β-E treatment. J. Cell. Biochem. 79:407–415, 2000.

Key words: ovariectomy; estrogen treatment; bone marrow cell differentiation

Loss of gonad function causes metabolic changes that are associated with a reduction in bone mass caused by a yet undefined mechanism. The decrease in estrogen and progesterone levels in vivo is characteristic of the postmenopausal state, aging, and surgical ovariectomy (OVX). Such conditions alter differentiation of the bone marrow cells (BMC), including the hemopoietic and stromal cells. Alteration in differentiation of the bipotential marrow stromal cells (MSC) to osteoblast or adipocyte was observed during aging or after OVX and results in changes of bone turnover [Beresford et al., 1992; Nuttall et al., 1998, Parhami et al., 1999, Park et al., 1999; Jaiswal et al., 2000]. The increase in adipocytes in the bone marrow is associated with thinning of trabecular bone, and leads to bone atrophy [Cirotteau, 1999; Li et al., 1999; Martin and Zissimos, 1991; Nuttall et al., 1998].

The study of aging or OVX in an animal model identified depletion of the mesenchymal progenitors and mature cells in the osteogenic lineage [Egrise et al., 1992; Kahn et al., 1995, Quarto et al., 1995, Liu et al. 2000]. The stroma cells are part of the hematopoietic inducible microenvironment (HIM). There is still some uncertainty concerning the ramifications of gonadal hormone intervention in the HIM that affects the differentiation and function of immune cells. This is an open question of prime importance involved with acute hormonal withdrawal or when the hormones are replaced by therapy. In this study, we sought to inves-

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tigate the effects of gonad hormones on the bone–marrow relationship and cell differentiation. Using histology, we were able to visualize the bone structural changes. Furthermore, we analyzed cell differentiation using flow cytometric analysis (FACS) and quantified cells of the immune system. We measured the myeloid lineage expressing ED-9 or CD-44 and the lymphoid cells expressing CD-4 or CD-5 surface antigens. The hormonal effects on cell differentiation were studied across skeletal maturation reflected by change in age and after OVX, with or without replacement therapy of 17β-estradiol (17β-E), progesterone (P), or both (17βE + P).

MATERIALS AND METHODS

Animals and Experimental Design

This study was designed to investigate the effect of age or hormonal changes on bone marrow structure. The experimental model used Fischer 344 rats (F344, 90-day and 11-month-old) (Animal Laboratories, Tel-Aviv University, Israel, A501001, and National Institute of Health welfare assurance). The total number of rats in the experiment was 120, each group containing 13–18 rats of both sexes. Operated female rats were subdivided into a control sham group (abdominal midline incision) and ovariectomized (OVX) group (bilateral ovariectomy performed with an abdominal midline incision) of 3- and 11-month-old animals. On the day of operation, the 11-month-old OVX rats were divided into three experimental groups that were implanted with 90-day slow-release pellets (Innovative Research of America, Sarasota, FL). Hormone replacement therapy of OVX rats with 17β-E, P, or 17β-E+P was compared to untreated OVX or sham rats. Serum levels of 17β-E (induced by 0.025-mg pellet) were elevated in serum to 40 pg/ml (physiological levels in cycling F344 are 0–80 pg/ml) and P (induced by 150-mg pellet) elevated serum levels to 20 ng/ml (physiological levels in cycling F344 are 5–50 ng/ml). At the end of the experiment, animals were killed and femurs were collected for further analysis.

Bone Marrow Cell Preparation

Femurs from each rat were cleaned off the soft tissue and epiphysis to allow BMC collection. The BMC were flushed out with Dulbecco's Modified Essential Medium (DMEM) using an 18-gauge needle pellet in a tube, and prepared as single cells suspension. Cells were washed in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) and analyzed by flow cytometry. Because of the large number of samples, the experiments were performed in three repeats on following days.

Antibodies

Antibodies were used to distinguish various lineages of lymph-myeloid cells: fluorescein (FITC)-conjugated anti-CD4 (OX-35), a marker for T-helper cell thymocytes, and phycoerthrin (PE)-conjugated-anti-CD5 (OX-19), a marker for thymocytes and peripheral lymphocytes (PharMingen, San Diego, CA). Mouse-(IgG1)-anti-rat-ED9 is a marker for macrophages, monocytes, dendrite cells, and granulocytes and mouse-(IgG2a)-anti-rat-CD44 (OX-49), a marker for macrophages and polymorphs (Serotec, Oxford, England). A second antibody, FITC-conjugated goat-anti-mouse (Sigma, Israel), was used to strain and separate positive cells.

Flow Cytometry–Immunofluorescence Analysis

BMC were divided in 96-well v-shaped-bottom plates 1×10^6 cells per well and then incubated with the specific antibody. Cells were incubated with each antibody for 30 min in room temperature (RT), then washed twice and resuspended in 1% paraformaldehyde in PBS containing 1% BSA and 0.01% azide. In case the first antibody was not directly labeled with fluorescent dye, a second fluorescent antibody label was used. The negative control was stained with second antibody only. Stained cells were analyzed on a flow cytometer (FACSort, Becton-Dickinson, San Jose, CA).

Histology

Femurs were fixed in 4% formalin in PBS and processed by conventional paraffin embedding. The sections were stained with hematoxylin/eosin and visualized by Nikon microscope.

Statistical Evaluation

The FACS analysis was calculated and the mean and SD was compared between subgroups using the analysis of variance method. Results considered significant for P values of < 0.05.

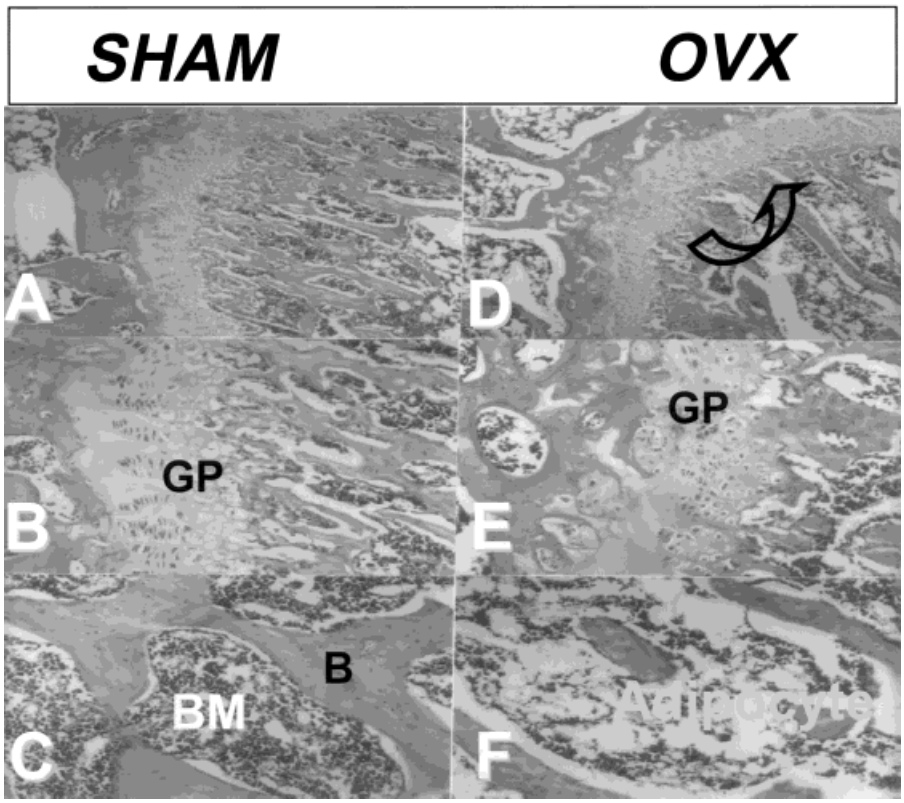

Fig. 1. Histology of bone structure and the bone marrow (BM) cellularity was compared between sham (**A–C**) and ovariectomy (OVX) (**D–F**) in 6-month-old rats. In these sections, a functional growth plate (GP) zone was visualized with proliferating and hypertrophied chondrocytes in lacunae. The growth plate is associated with trabeculae bone that is extended toward the bone marrow in the medullary cavity. Visible mass and extended bone trabeculae were seen in the sham group that appeared fragile in the OVX rats (arrow, D). Increase in adipocyte in OVX is visualized (F). Magnification in A, D: ×40; B, C, E, F: ×100.

RESULTS

We used a rat model to elucidate the effects of hormonal changes on bone structure and cellular composition of BMC; hematopoietic and stromal cells. We analyzed sham and OVX 6- and 14-month-old rats at 3 months after surgery. Histology was used to demonstrate the changes in bone structure and the bone marrow cellularity makeup. In the young age group (6 months old), a clear functional growth plate zone with proliferating and hypertrophied chondrocytes was visualized (Fig. 1A,B), whereas the growth plate of mature rats (14 months old) was not active (Fig. 2A). The growth plate zone is associated with trabeculae bone that is extended toward the medullary cavity. The bone trabeculae were more developed in terms of total mass in the sham group at both ages (Fig. 1A–C and Fig. 2A–C) as compared to the OVX ones. The trabecular bone appeared fragile in the OVX rats at both ages (see arrow in Fig. 1D and Fig. 2D), but this phenomenon was more prominent in the older age group (Fig. 2D–F). Moreover, changes in the anatomical organization of bone marrow cells were observed in the OVX (Fig. 1F and Fig. 2F) when compared to the sham group (Fig. 1C and Fig. 2C). Less nucleated cells in the medullary cavity of OVX rats were observed and was associated with a greater number of yellow adipocytes (Fig. 1F and Fig. 2F). Blood vessels at the medullar cavity were prominent observed in aged rats (Fig. 2F). In summary, changes in the bone and bone marrow structure were observed in the 14-month-old rats (sham or OVX) (Fig. 2) in comparison to the young group (Fig. 1). An increase in numbers of adipocytes with age and when gonad hormones were depleted in OVX rats was also

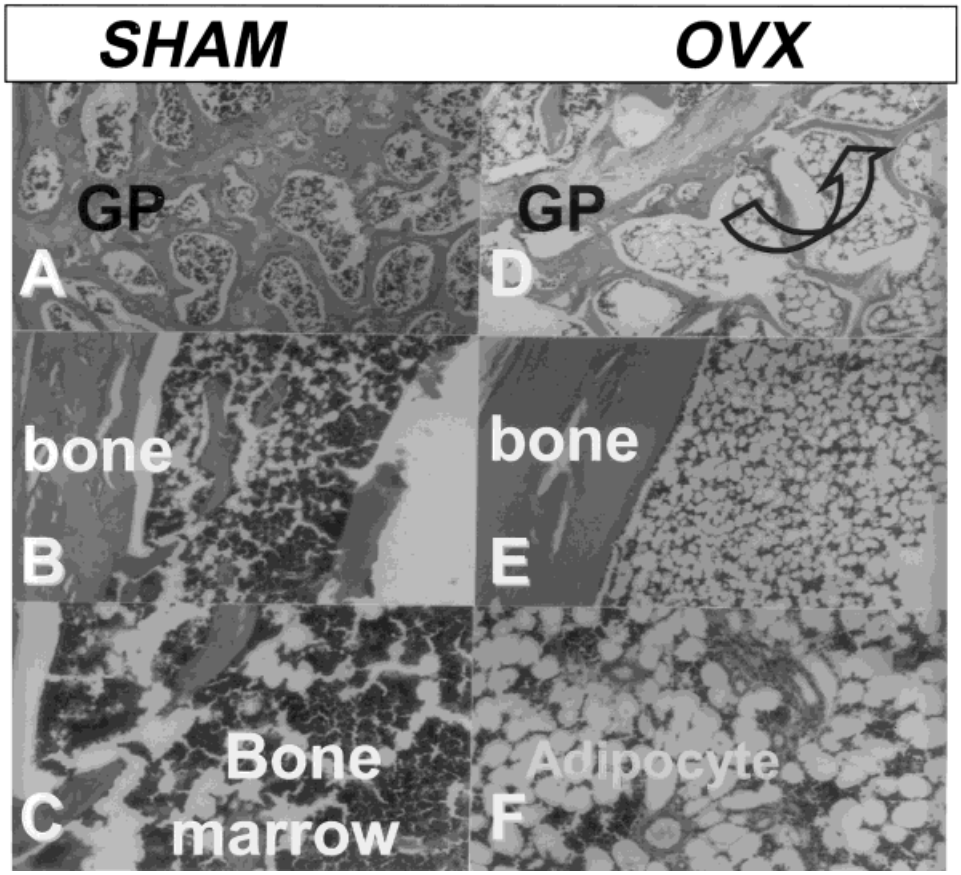

Fig. 2. Histology of bone structure and the bone marrow cellularity makeup was compared between sham (**A–C**) and in ovariectomy (OVX) (**D–F**) in 14-month-old rats. In these sections, a growth plate (GP) zone is not active. The comparison of 14-month-old rats (sham or OVX) results in bone trabeculae appearance that developed as a net form. The trabeculae appeared fragile in the OVX rat (see arrow, D). The bone marrow appearance revealed a different pattern, with a significantly greater number of adipocytes in the medullary cavity of OVX rats (E–F). Magnification in A, D: ×40; in B, C, E, F: ×100.

associated with thinning of structure of the trabecular bone.

FACS analysis used to quantify the number of lymph-myeloid cells and to compare between the experimental groups based on age, gender, and hormonal background. We quantify the myeloid cells with antibodies to CD-44 and ED-9 (Figs. 3 and 4) and T-lymphoid cells were analyzed with antibodies to the CD-4, CD-5 surface markers (Fig. 5). The myeloid cells analyzed by the expression of CD44 and ED-9 surface markers were compared between the young (6-month-old/14-month-old), male/female and sham/OVX groups. The analyzed cells expressed 40%–54% $CD44^+$ (Fig. 3A) and 50%–65% that were $ED\text{-}9^+$ (Fig. 4A). The same pattern of expression was observed in experimental groups of both ages or when compared between male and female or based on hormonal status (sham or OVX groups).

At the second phases of the experiment, we followed the cell differentiation of 14-month-old rats that were OVX or treated with replacement therapy of 17β-E, P, or combined 17β-E+P. The basal level of positive myeloid cells determined by the expression of ED-9 or CD-44 was unchanged between the sham group when compared to OVX or treated rats with 17β-E, P, or combined 17β-E+P (Figs. 3B and 4B). FACS analysis for T-lymphoid cells was quantified using antibodies to CD4 and CD5 surface markers. We compared the T cells between old/young, and sham/OVX rats. An age-related increase in cell number of T-lymphoid subpopulation was observed for $CD\text{-}4^+$, $CD\text{-}5^+$, or both (Fig. 5). The 6-month-old rats had lower $CD\text{-}4^+$, $CD\text{-}5^+$, or both cells than the sham of 14-month-old rats (Fig. 5A, Table I). The T cells revealed an upregulation in OVX state and with age (Fig.

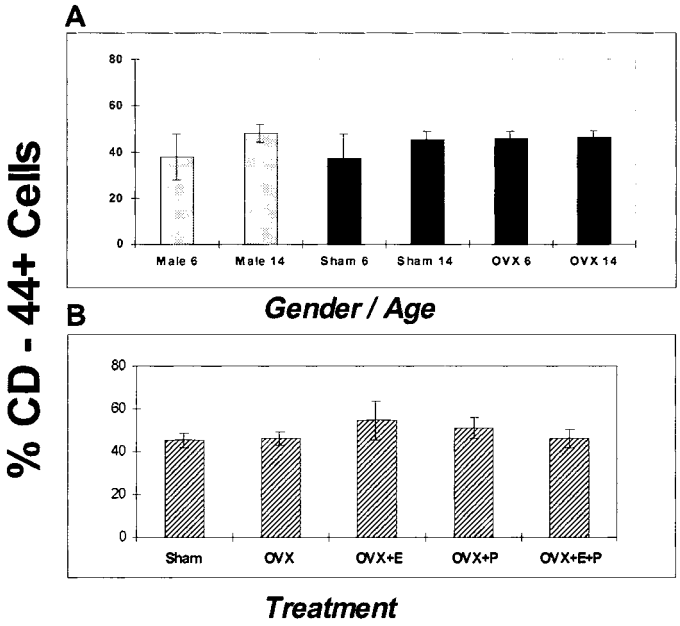

Fig. 3. Myeloid cells stained with antibody to CD44 analyzed by flow cytometry to compare between (**A**) experimental groups, based on age, gender: male, female that were sham, ovariectomy (OVX) of 6- and 14-month-old rats and (**B**) 14-month sham, OVX, OVX-treated rats (17β-estradiol: 17β-E, progesterone: P, or combined treatment: 17β-E+P). The percentage of positive cells measured is summarized by mean ± SD of 10–13 rats per group.

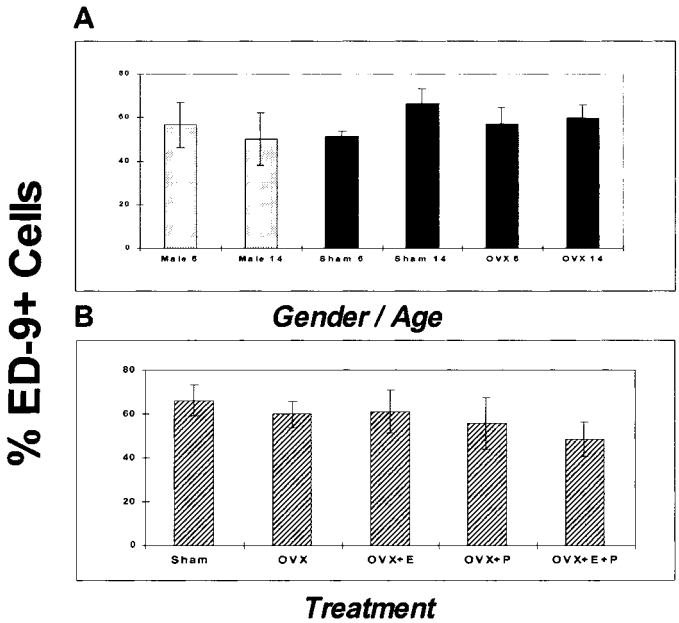

Fig. 4. Myeloid cells stained with antibody to ED9 analyzed by flow cytometry to compare between (**A**) experimental groups, based on age and gender: male, female that were sham, ovariectomy (OVX) of 6- and 14-month-old rats and (**B**) 14-month-old sham, OVX, OVX-treated rats 17β-estradiol (17β-E), progesterone (P), or combined treatment (17β E+P). The percentage of positive cells measured is summarized by mean ± SD of 10–13 rats per group.

5A). The CD-4^+ cells were increased between the OVX of young/old rats and compared to sham rats of both age groups ($P < 0.04$ for CD-4^+ and $P < 0.012$ for CD-5^+) (Fig. 5A). The T-cells level of 14-month-old OVX rats was compared to OVX-treated with replacement therapy of 17β-E, P, or combined 17β-E+P or sham group. The administration of 17β-E to OVX rats decreased the T cells to sham level (Fig. 5B). OVX-P treatment did not alter the number of cells from untreated OVX, whereas the combined 17β-E+P treatment decreased the T-cell type to sham level. The CD-5^+ and CD-4^+/CD-5^+ OVX rats were also changed in their cell number with the same profile after 17β-E+P treatment but not when progesterone-treated alone (Fig. 5B). In conclusion, an upregulation of T-lymphoid cells in the OVX rats was measured in comparison to the sham group at both ages and reversed to baseline level by 17β-E.

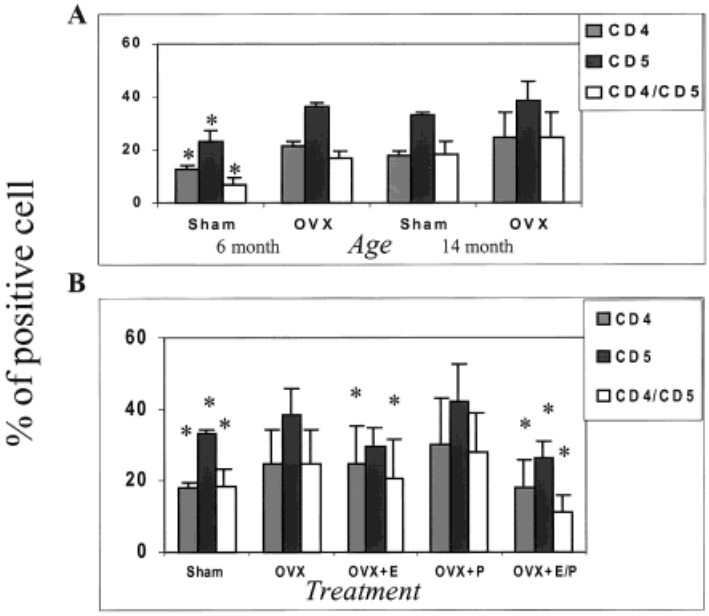

Fig. 5. T-lymphoid cells staining to CD4 and CD5 expression analyzed by flow cytometry to compare between (**A**) experimental groups: sham or ovariectomy (OVX) of 6- and 14-month-old age rats (**B**) 14-month-old rats compared the sham, OVX, with OVX-treated rats (17β-estradiol: 17β-E, progesterone: P, or combined treatment: 17β-E+P). The percentage of positive cells summarized as mean ± SD for 10–13 rats per group and significant is marked by asterisks between subgroups.

TABLE I. Lymphoid Cells Analyzed by Antibody Staining for the Expression of CD-4 and CD5[a]

Group	Mean ± SD CD 4+	Mean ± SD CD 5+	Mean ± SD CD4+/CD5+
6-month			
Sham	13.6 ± 1.5	21.7 ± 2.8	9.6 ± 1.3
OVX	19.1 ± 3	34.1 ± 2.5	14.8 ± 3.1
14-month			
Sham	17.7 ± 7.3	26.8 ± 6	14.9 ± 6.9
OVX	24.2 ± 8.2	37.7 ± 4	20.6 ± 5.7
OVX + E	25.2 ± 6.4	31.9 ± 5.3	22.3 ± 6.5
OVX + P	30.2 ± 7.5	43.2 ± 6.8	28.5 ± 9.3
OVX + E/P	15.6 ± 6.6	23.6 ± 3.8	11.5 ± 4.1

[a]OVX, ovariectomy; OVX + E, OVX with 17β-estradiol; OVX + P, OVX with progesterone; OVX + E/P, OVX with 17β-estradiol and progesterone.

DISCUSSION

The gonad hormone deficiency changed cells differentiation that are associated with bone loss. We observed histologically an alteration with age in bone trabecular structure and in the growth plate of OVX rats from the sham rats. These changes result from reduction in osteoblastic activity associated with an increase in adipocytes in the bone marrow. It was reported earlier that a decline of mesenchymal stem cells pool in estrogen depleted animal led to a decrease of bone formation [Egrise et al., 1992; Kahn et al., 1995; Quarto et al., 1995]. Replacement therapy (estrogen or selective estrogen analogs) could prevent this bone loss in the mouse and rat models [Schwartz et al., 1991; Ornoy et al., 1994; Black et al., 1994; Evans et al., 1994; Sato et al., 1994; Turner et al., 1994; Li et al., 1998; Arjmandi et al., 2000]. It is believed that estrogen directly affects bone remodeling by inhibition of osteoclast activity, which also affects stromal stem cell differentiation to mature skeletal cells. The osteoblasts and chondrocytes express the estrogen receptor in rat, mouse, and human models [Eriksen et al., 1988; Ben-Hur et al., 1993; Pinus et al., 1993; Nasatzky et al., 1994; Komm et al., 1988; Shamay et al., 1996]. The estrogen affects various cellular activities such as protein synthesis, ALK-P activity [Shamay et al., 1996], and cytoskeleton organization of cultured osteoblasts [Benayahu, 1997]. A fast effect was also monitored on osteoblastic cell communication and changes of gap junction formation [Massas et al., 1998; Schirrmacher and Bingmann, 1998].

The pathophysiology of gonad deficiency is associated with bone loss, deterioration of the bone structure, and affects cells of the immune system. A considerable volume of evidence points at age and hormonal differences on immune responsiveness. Sex hormones act as negative regulators on lymphopoiesis differentiation in culture and in vivo. A rat model was used in this study to measure the alteration in

number of lymph-myeloid cell differentiation. An augmentation in lymphopoiesis of CD-4^+ and CD-5^+ cells was noted and correlated to age or hormonal background. Treatment of OVX rats (14-month-old) with replacement therapy (17β-E or 17β-E+P) decreased the lymphoid cells to sham level. The role of hormones in the immune system demonstrated an inverse relation between estrogen levels and subset of lymphocyte cells of increased CD-8^+ and natural killer cells and a decrease of B cells [Giglio et al., 1994]. Higher levels of CD-4^+/CD-8^+ were measured in osteoporotic patients [Fujita et al., 1984; Imai et al., 1990]. However, the pattern of lymphoid cells is not uniform; some studies with osteoporotic women could not confirm these changes reported by the latter [Hustmyer et al., 1993]. The activated and elevated level of T cells was suggested to occur in response to increased levels of inflammatory cytokines in the circulation. Activated T cells can regulate systemic and local bone loss as was shown recently through osteoprotegerin ligand [Kong et al., 1999]. Treatment with different bisphosphonates shows differential effects with benefit of pamidronate to be negative regulator of subset of T-cells [Pecherstorfer et al., 2000].

Sex hormones differentially affect lymphopoiesis or myelopoiesis. It was demonstrated in the mouse model that shortly after OVX surgery, B-lymphocytes (B-220^+) were selectively increased, and myeloid cells were decreased or did not change appreciably. Studies show that deficiency of sex steroids increased B lymphopoiesis and bone loss, whereas estrogen and raloxifene prevented this increase [Kincade et al., 1994; Masauzawa et al., 1994; Onoe et al., 2000]. In other mouse models, when OVX existed for longer periods, no changes in B lymphophoiesis were observed [Liu et al., 2000]. In 3-month-old rats the lymphoid cells, that quantified for Thy1.1 co-expression with B-cell markers, were increased over sham level [Erben et al., 1998 a,b). Progenitors of nonlymphoid cells are regulated differently, and it was shown that myeloid cells were increased when analyzed both by granulocyte macrophage colony-forming units colony formation or by FACS of Mac-1^+, Gr-1^+ antigens. The upregulation of myeloid cells was lowered after administration of raloxifene [Liu et al., 2000] or estrogen [Jilka et al., 1992, 1995]. The alteration in myeloid differentiation leading to upregulation of osteoclastogenesis is believed to be mediated via increased interleukin-6 levels when estrogen is depleted from the circulation [Jilka et al., 1995]. In the present study, we measured the unchanged frequency of myeloid cells expressing CD-44 and ED-9, in the OVX group compared to the sham ones.

In different OVX models, the changes in the hemopoietic population were not expressed with a similar pattern. The differences between experimental models may be because of the retention of OVX period and the type and period of treatment after the OVX. In summary, the present study demonstrated bone loss in OVX of 6- and 14-month-old rats, when compared to the sham ones. We also quantify that myeloid cells respond differently from the lymphoid cells with age and when the hormonal background was altered. The results show that changes associated with aging or hormonal depletion affect both the skeleton and the immune system. This study adds valuable information on cell differentiation in the complex skeletal–bone marrow microenvironment.

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