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Effect of Raloxifene-Analog (LY 117018-Hcl) on the Bone Marrow of Ovariectomized Mice

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Abstract The effects of LY117018-Hcl (Ralox-A) on body metabolism and differentiation of bone marrow cells were studied in ovariectomized (OVX) mice. We used a mouse model in which estrogen depletion was established for a period of three months before treatment. After that period the animals were divided into three experimental groups consisting of sham-operated, OVX, and OVX-Ralox-A-treated mice. The OVX animals received daily treatment of Ralox-A during two time periods (35 and 65 days). After the treatment we measured the serum levels of protein, ion(s), lipid content, liver, and kidney functions. Our findings indicated that a change in hormonal state did not affect basic body metabolism except for causing an increase in triglycerides (TG) in the OVX mice, which was lowered by the Ralox-A. A higher alkaline phosphatase (ALK-P) level was observed in serum of the OVX-Ralox-A-treated mice than in serum of the OVX mice. We investigated the effects of estrogen depletion on the differentiation of hematopoietic and stromal cells that directly affect bone resorption and formation. OVX and OVX-treated mice were compared with the sham group and assessed for the alteration of these cells' differentiation. The proliferation of stromal stem cells was measured by CFU-F assay in vitro. A decrease in CFU-F colonies derived from OVX mice was observed and after the Ralox-A treatment the number of CFU-F reached sham levels. On the contrary, an upregulation of myeloid cells was observed when analyzed by FACS and by granulocyte/macrophage–colony forming unit (G/M-CFU) assay in selective culture conditions. The G/M-CFUs were increased in the OVX mice and were reduced to sham levels after Ralox-A treatment. In this study, we demonstrated cellular changes of stromal and hemopoietic cells in OVX mice and a beneficial Ralox-A effect that protected such cellular changes. J. Cell. Biochem. 76:509–517, 2000.

Key words: ovariectomy; estrogen depletion; raloxifene-analog; bone marrow cell differentiation

Osteoporosis is a metabolic bone disorder characterized by a significant loss of bone mass. This condition observed at a postmenopausal state or following ovariectomy (OVX). It is speculated that estrogen depletion is a major reason for the development of the disease. This has led to the consideration and use of estrogen as replacement therapy to protect against bone loss. However, undesired uterotropic effects are often associated with estrogen therapy. New classes of compounds have been developed that express a selective estrogen receptor modulation (SERM) activity. Antiestrogens including tamoxifen and raloxifene have in vivo effects and exhibit tissue-selective activity that is also based on the identification of estrogen response elements (EREs). In bone, certain key regulatory elements are mediated through pathways independent of EREs. These explain the antiestrogen agonists that demonstrate a distinct ligand preference [Yang et al., 1996]. The SERMs were beneficial agonists on bone and in the cardiovascular system [Sato et al., 1996; Ward et al., 1993] and were antagonists on the uterus and breast. Thus, the differential activity helps to overcome the undesired uterotropic effects often associated with estrogen-related therapies. SERMs are now receiving major attention in research and in the clinic, and are being used to protect against postmenopausal bone loss.

Cellular changes associated with estrogen depletion involved with the alteration of differentiation of hemopoietic cells that are osteoclast precursors explain the increased bone resorption. Osteoprogenitors derived from the

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stromal cells [Benayahu et al., 1989, 1991; Owen and Friedenstein, 1988]. A decline in osteoprogenitor maturation in low estrogen levels and aging is recognized and led to a reduction in bone mass and bone repair potential [Egrise et al., 1992; Kahn et al., 1995; Quarto et al., 1995]. There is no complete knowledge regarding the regulation of these stromal and hemopoietic progenitors' differentiation and the hormonal effects, especially estrogen.

The SERMs are increasingly used for osteoporosis or breast cancer treatment and their effects on the immune system, especially on cells of the bone marrow, have not been explored yet. In the present study, we used raloxifene-analog (Ralox-A, LY 117018-Hcl) that was originally characterized as SERM based on its ability to inhibit binding of 17-β-estradiol (E_2) to the estrogen receptor [Black et al., 1994; Evans et al., 1994; Sato et al., 1994; Turner et al., 1994; Whitfield et al., 1995]. Our attempt was to elucidate the regulation of Ralox-A, 117018-Hcl, in OVX-mice model. We used OVX mice that were treated with the drug to assay its effect on body physiology measured by biochemical blood parameters. We also monitored the drug's effects on the bone marrow compartment for differentiation of hemopoietic and stromal progenitors that produce the mature cells active in bone remodeling and the immune system.

MATERIALS AND METHODS

Animal and Experimental Design

This study was designed to investigate the effect of LY 117018 Hcl (Ralox-A) on hemopoietic and stromal cell differentiation in OVX mice. Our experimental model used 90-day-old Balb/C mice (Animal Laboratories, Tel-Aviv University, Israel, A501001, NIH welfare assurance). All mice were anesthetized with pentobarbitone sodium (30 mg/kg) and divided into a sham-operated (control) group and an ovariectomized (OVX) group (bilateral ovariectomy was performed with a midline abdominal incision). In the sham-operated animals, the ovaries were taken out and then returned to the abdomen. Our working hypothesis was to study the drug's effects in established gonadal hormone deficient mice. For this, we allowed a maintenance period of 3 months before the OVX mice received the treatment.

In order to evaluate the LY 117018 Hcl Ralox-A effects on the OVX mice they were analyzed at two periods post treatment starting day; 35 days (group A, Experiment 1) and 65 days (group B, Experiment 2). Thus, the study contained three experimental groups: sham operated (control), OVX, and OVX-treated with Ralox-A (LY 117018 Hcl; Lilly Research Laboratories, Indianapolis, IN). The control and OVX mice groups received solvent vehicle by daily subcutaneous injection and the OVX-treated group were injected subcutaneously with the drug (1 mg/kg). Each experimental group contained 10–13 mice. On the last day of each experiment, the animals were killed and blood and tissue samples were collected for further analysis.

Serum Collection and Bioassay

The mice were bled from a retroorbital plexus for whole blood collection. Blood samples were allowed to clot overnight at 4°C and then centrifuged at 2,500*g* for 10 min. Serum samples were separated and stored at −20°C for analytical procedures.

Blood chemistry analyses were performed by using an automatic analyzer (Boehringer-Hitachi 747). The parameters were examined with Boehringer Mannheim analytical kits including those for kidney function (measured by blood urea nitrogen [BUN, mg/dL] and serum uric acid [UA, mg/dL]) and liver function (measured by glutamic oxaloacetic transaminase [GOT, AST, IU/L] and glutamate-pyruvate transaminase [GPT, ALT, IU/L]). Protein was measured by total protein (TP g/L), albumin (g/L), globulin (calculated, g/L), and albumin: globulin ratio. Serum alkaline-phosphatase (ALK-P), inorganic phosphorus (Pi), total calcium (Ca), and magnesium (Mg) were measured with analytical kits (Boehringer Mannheim). Serum cholesterol was determined with a high-performance cholesterol colorimetric assay. Serum triglyceride was determined by an enzymatic method. Serum high-density lipoprotein (HDL-C) was determined by precipitation with phosphotungstate with a kit (Boehringer Mannheim).

In Vitro Bone Marrow Cellularity Study

Bone marrow cells (BMC) were collected from femurs for further in vitro analysis.

Colony forming unit-fibroblast (CFU-F). The BMC suspension was diluted to a concentration of 2.5×10^6 cells/ml in DMEM supplemented with 10% FCS. One-ml aliquots of these

suspensions were inoculated into 30-mm culture dishes to which a further 1-ml of standard medium was added; they were then incubated for 7 days. Primary cultures were scored with an inverted microscope for determining the number of CFU-F colonies. Six to 10 culture dishes were plated for each mouse.

Granulocyte/macrophage (G/M)-colony forming unit (GM-CFU). BMC single cell suspensions were adjusted to a concentration of 4×10^5 cells/ml in Dulbecco's minimal essential medium (DMEM) supplemented with 20% FCS and murine interleukin-3 (mIL3, Genetic Institute). Cells were seeded in 0.8% (W/V) methylcellulose, and 1-ml aliquots of these suspensions were inoculated into 30-mm culture dishes (triplicate cultures for each mice) and incubated for 8 days, then myeloid colonies were counted by an inverted microscope.

Flow Cytometric Analysis

Cell surface staining. 10^6 bone marrow cells were incubated with antibodies for myeloid cell antigens αGR-1 (granulocytes), αMAC-1 (macrophages), or towards pre-B cells (TIB164), using hybridoma supernatants (from the American Type Culture Collection, ATCC) for 90 min, on ice with occasional agitation. The cells were then incubated with goat anti-mouse FITC conjugated secondary antibody (Zymed) for 30 min and washed in PBS, and a sample of labeled cells was analyzed.

Nile Red staining. 10^5 bone marrow cells were stained with Nile Red dye (Sigma) as described by Dorheim et al. [1993]. Gold fluorescence emission was detected between 564 and 604 nm and was analyzed.

For both analyses we used a Beckton Dickinson FACS.

Statistical Evaluation

The statistical calculations as means and standard errors of the mean was analyzed by analysis of variance (ANOVA). Results of the experimental group were compared to the sham group by the ANOVA multiple comparison test.

RESULTS

Blood Chemistry Parameters

The effects of OVX and OVX-Ralox-A-treated mice on hepatic (GPT, GOT) and renal (BUN, UA) function were compared to sham-operated mice (Table IA). Serum content of total protein, albumin, globulin, and albumin/globulin ratio (Table IB) was comparable in the three experimental groups and used to reflect the health of the animals. The basic physiological function was not different in the parameters examined at the end of both experimental periods (Exp. 1, 35 days; and Exp 2, 65 days) of the treatment. Additional measurements of ion(s), such as Ca, Pi, and Mg in the serum of three groups of mice, were also unchanged (Table IC). All parameters presented here indicate that the drug had no toxic effects on the basic physiology of the experimental mice.

Serum lipids measured by cholesterol, TG, and HDL were determined in three experimental groups (Fig. 1). Cholesterol and HDL were not significantly different between the three groups at two time points examined. The elevation of TG levels was observed in the OVX mice with the prolongation of time (Exp. 2, B) and Ralox-A-treated OVX mice were decreased to sham-operated levels ($P < 0.05$, Fig. 1).

Serum ALK-P enzymatic activity was measured at both time points of the treatment. The enzyme levels of OVX and OVX-Ralox-A treated mice were unchanged from that of the sham group following 35 days (Fig. 2, Exp. 1). However, it is shown that with prolongation of the treatment after 65 days in the OVX mice an increase in ALK-P level was measured. This indicates an increase in bone formation activity in the Ralox-A-treated mice (Fig. 2, Exp. 2, $P < 0.05$).

Bone Marrow Cell (BMC) Cellularity

We studied the differentiation of stroma (Figs. 3 and 4) and hemopoietic cells (Figs. 5 and 6) and compared the cellular changes between sham-operated, OVX, and OVX-Ralox-A-treated mice. The pool of progenitor cells from the bone marrow was analyzed in two treatment periods: Exp. 1 for 35 days and Exp. 2 for 65 days.

Stromal compartment. It is now thought that osteoprogenitors differentiate from mesenchymal precursor cells and form in vitro the fibroblastic colony forming unit (CFU-F) from bone marrow cells. The availability and activity of these cells may have a profound effect on bone formation. We studied the capacity of stromal cells to proliferate and to form CFU-F colonies. The number of colonies formed was controlled by the hormonal state of the mice. The hormonal effect in vivo was measured on growth capacity of the progenitor cells' pool. It

TABLE I. Blood Chemistry Results[a]

	A. Kidney and liver function			
	BUN (mg/dL)	UA (mg/dL)	GPT (IU/L)	GOT (IU/L)
Exp. 1				
S	34.5 ± 4.96	6.1 ± 1.9	19.75 ± 5.35	127.25 ± 33.25
O	30.4 ± 4.29	6.2 ± 2	21.25 ± 4.46	123.55 ± 27.4
R	30.2 ± 8.47	6.6 ± 1.3	18 ± 3.7	109 ± 22
Exp. 2				
S	33 ± 5.86	3.9 ± 1.5	30 ± 9.66	123.3 ± 24.65
O	29.4 ± 5.09	4 ± 1.4	24.9 ± 3.3	101.8 ± 16.7
R	29 ± 3.46	4.5 ± 2.1	33.8 ± 17.2	112.2 ± 26.54

	B. Protein content			
	TP (g/L)	Albumin (g/L)	Globulin (g/L)	A/G
Exp. 1				
S	54 ± 3	29.5 ± 1.74	24.5 ± 3.03	1.22 ± 0.15
O	58 ± 5.7	28.1 ± 2.00	29.8 ± 6.13	1.68 ± 2.41
R	55 ± 4.5	28.8 ± 2.66	25.9 ± 3.38	1.12 ± 0.15
Exp. 2				
S	55 ± 4	27.6 ± 1.6	27.4 ± 3.15	1.03 ± 0.11
O	55 ± 3.5	27.7 ± 1.49	27.2 ± 2.73	1.03 ± 0.12
R	55 ± 2.8	28.8 ± 2.05	25.8 ± 1.64	1.13 ± 0.1

	C. Serum ions		
	Ca^{++} (mg/dL)	Pi^{+++} (mg/dL)	Mg^{+++} (mg/dL)
Exp. 1			
S	9.39 ± 0.36	8.22 ± 1.3	3.1 ± 0.2
O	9.87 ± 0.5	8.62 ± 1.66	3.1 ± 0.4
R	9.52 ± 0.42	9.85 ± 2.31	3.1 ± 0.5
Exp. 2			
S	9.73 ± 0.29	8.8 ± 1.11	3.4 ± 0.2
O	9.64 ± 0.39	8.48 ± 1.8	3.3 ± 0.3
R	9.68 ± 0.31	8 ± 1.23	3.2 ± 0.2

[a]Blood chemistry analyses were performed by using Boehringer-Mannheim kits and measured by automatic analyzer as detailed in Materials and Methods. S, sham-operated; O, ovariectomized; R, ovariectomized Ralox-A-treated. Each group of animals included 10–13 mice at two time points: The Ralox-A treatment in vivo was continued throughout 35 days (A, Exp. 1) and 65 days (B, Exp. 2). No difference was observed between groups.

was measured by the in vitro CFU-F assay (Fig. 3). Primary cultures of fibroblast colonies (CFU-F) from the three experimental groups were scored for their proliferation potential. CFU-F colony count following OVX showed a reduction in number in comparison to the sham-operated group. In experiment 1 they were not significantly lower, however, with prolongation of time the CFU-F in OVX mice were significantly lower than that of the sham group. At both time points, the number of CFU-F from OVX-Ralox-A-treated increased to the sham level ($P < 0.05$, Fig. 3). We demonstrated changes of CFU-F that are associated with the hormonal status of the animal.

The differentiation of stromal progenitors in vivo also measured the proportion of adipocyte population. An increase in Nile Red emission was consistent with adipogenesis. This was quantitated among the three experimental groups and directly reflected the number of the adipocytes in the bone marrow (Fig. 4). An elevation in fluorescence intensity was measured in the BMC of OVX mice in comparison to sham mice ($P < 0.015$). The increase of adipocytes in the bone marrow of OVX mice was

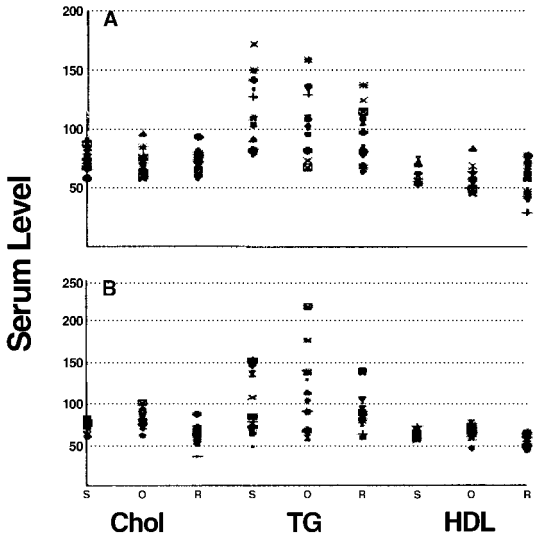

Fig. 1. The effect of Ralox-A on lipid profile in serum (cholesterol, TG, HDL) of ovariectomized (OVX) mice treated for two time periods: 35 days (Exp. 1, **A**) and 65 days (Exp. 2, **B**). Comparative results of sham (S), OVX (O), and OVX-Ralox-A-treated (R) mice are summarized. Each set represents the distribution of results for each group (n = 11–13). Statistical significance ($P < 0.05$) is between the OVX and sham or OVX-Ralox-A treated mice of TG analysis of Exp. 2, (**B**).

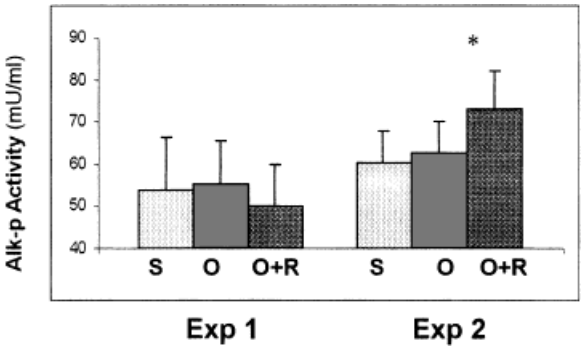

Fig. 2. Serum ALK-P activity from three experimental groups of mice was analyzed for the Ralox-A treatment for two periods of 35 days (Exp. 1) and 65 days (Exp. 2) of OVX mice. Comparisons of sham (S), ovariectomized (O), and OVX-Ralox-A-treated (O + R) mice are summarized (mean ± SD; (n = 13). There was a significant difference (*$P < 0.05$, Exp. 2) between OVX-Ralox-A-treated and other groups.

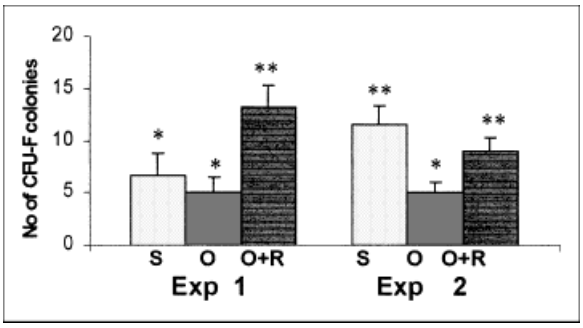

Fig. 3. CFU-F colonies grown in culture from BMC from the experimental groups (sham, OVX, and OVX-Ralox-A-treated mice) throughout 35 days (Exp. 1) and 65 days (Exp. 2). Six to ten plated dishes were counted for each mouse, and each group represents the mean ± SD of 11–13 animals. The comparison between groups of mean number of colonies, significant were marked with * or ** ($P < 0.05$).

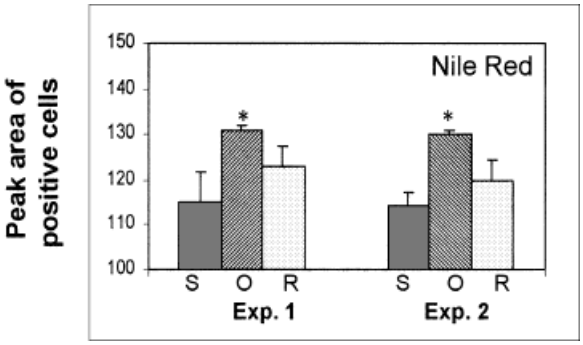

Fig. 4. To analyze the pattern of distribution of adipocyte cells from the BMC, cells were stained with Nile Red and read with a flow cytometer. The differentiation of adipocytes was calculated by the percentage of cells differentiated based on the total fluorescence that corresponds to the number of cells. The calculation of the three experimental groups, sham (S), OVX (O), and OVX-raloxifene-treated (R) summarize the mean for four to six mice per group; they significantly differ between sham group and OVX ($P < 0.015$).

reduced in the Ralox-A-treated mice (Fig. 4). This experiment followed changes of cellular differentiation and their make-up in the bone marrow that are affected by the hormonal (estrogen or SERMs) levels.

Hemopoietic compartment: Committed myeloid G/M progenitors. Using the GM-CFU assay in selective culture conditions we monitored the changes of myeloid cell differentiation. This assay measured the cellular differentiation in OVX mice and compared to OVX-Ralox-A treated or to sham-operated mice. We were able to demonstrate an increase in the numbers of committed GM-CFU cells in OVX mice: 129% in Exp. 1 (Fig. 5A and Table II) and 170% in Exp. 2 (Fig. 5B and Table II), the latter is statistically significant ($P < 0.001$) when compared to the sham level. The number of GM-CFU was lowered in the OVX-Ralox-A-treated group. Significant differences were observed in the Ralox-A-treated mice in Exp. 2 compared to OVX mice and are indistinguishable from the sham group, which means that these mice expressed the same potential to form GM-CFU colonies.

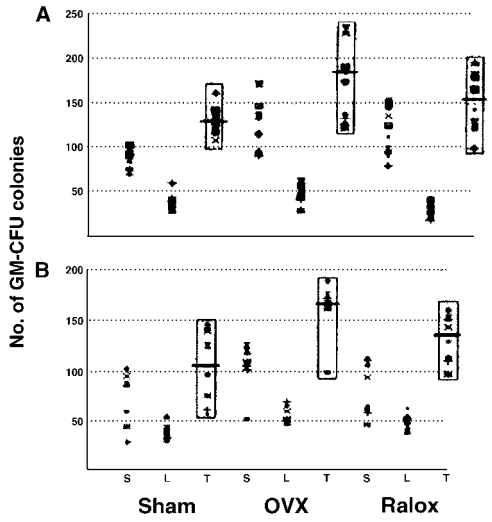

Fig. 5. GM-CFU colonies grown in semisolid culture from BMC (as described in Materials and Methods) from three experimental groups, sham (S), OVX (O), and OVX-Ralox-A-treated (Ralox) mice. Raloxifene treatment in vivo was continued throughout 35 days (**A**; Exp. 1) and 65 days (**B**; Exp 2). Colony counts for each group represent 13 separate animals divided in (S) small colonies< then 50 cells, (L) large colonies > then 50 cells, and (T) total number that combine S + L. The mean number of colonies is represented by (-). A significant increase in GM-CFU colonies at OVX group over the sham and OVX-Ralox-A treated mice is observed with prolonged time (Exp. 2B; $P < 0.001$).

Myeloid and lymphoid cells' differentiation in the bone marrow. We used the flow cytometric analysis for BMC from the three experimental groups and measured specific antigen expression for myeloid and lymphoid cell lineage. The specific antibodies were used to determine the proportion of positive cells for macrophages (αMAC-1) or granulocytes (αGR-1) that are in the myeloid lineage (Fig. 6). An increase in the cell number of the G/M subpopulation was observed in OVX mice over the control level at two time schedules. The increase was statistically significant for two subpopulations examined with significant value $P < 0.02$ for Exp. 1 and $P < 0.05$ for Exp. 2. The in vivo treatment of OVX animals with Ralox-A reduced the number of MAC-1+ve and GR-1+ve cells to sham level with prolonged time (Exp. 2, Fig. 6). The FACS analysis revealed complementary results of the measured granulocyte/macrophage cells, as assayed by the GM-CFU colony formation (Fig. 5).

B-lymphoid cells examined from the BMC of these mice were unaffected by changes in the metabolic state of OVX and OVX-Ralox-A-treated mice when compared to the sham-operated group (Fig. 6). These results indicate that the response of myeloid cells is different from that of the lymphoid subpopulations.

The experiment was designed to evaluate the increase in myeloid cells that are GM-CFU progenitors, also recognized as osteoclast precursors. The hormonal changes clearly affected

TABLE II. Total Number of GM-CFU From Three Experimental Groups[a]

Exp. 1	
S	130 ± 15.5*
O	167 ± 49.9**
R	152 ± 33.9*,**
Exp. 2	
S	107 ± 38.1*
O	171 ± 11.6**
R	129 ± 20.2*

[a]GM-CFU colonies grown in semisolid culture from BMC from three experimental groups: sham (S), OVX (O), and OVX-Ralox-A-treated mice (R). Raloxifene treatment in vivo was continued throughout 35 days (Exp. 1) and 65 days (Exp. 2). Total number of colonies that combined the small colonies (<50 cells) and large colonies (>50 cells) as detailed in Figure 5. The table presents the mean number of total colonies (*,**$P < 0.05$ comparing each asterisked value).

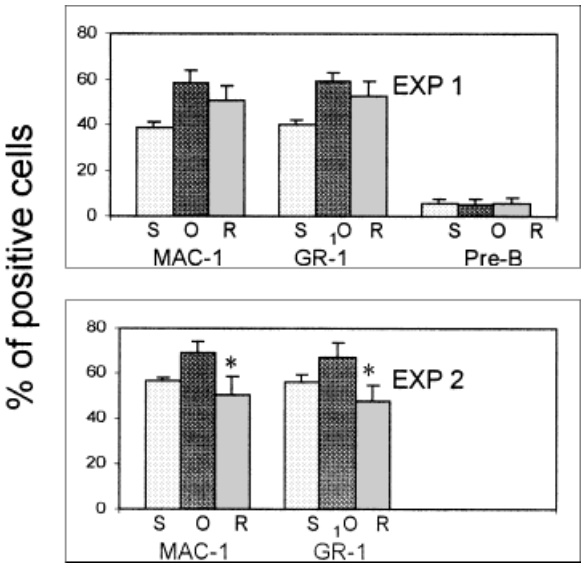

Fig. 6. Pattern of distribution of BMC staining with different MoAbs and sorted with a flow cytometer in the three experimental groups, sham (S), OVX (O), and OVX-Ralox-A-treated (R). Determinating the percentage of positive myeloid cells (αMAC-1), granulocytes (αGR-1), and pre-B lymphoid cells were measured. The results are the mean for four to six per group of mice, and a significant increase of cells in the OVX group over sham levels, that was then reduced by the therapy is demonstrated with a significant difference between OVX and OVX-Ralox-A in Exp. 2 with ($P < 0.05$).

the differentiation of the G/M precursor cells but did not affect the B-lymphoid cells.

DISCUSSION

Bone loss occurs in both postmenopausal women and ovariectomized (OVX) animals due to estrogen depletion. There is still some uncertainty regarding the nature of the pathogenesis of this disorder in terms of simple imbalances in calcitrophic hormones. Our interest was to analyze the direct action of gonadal hormones on bone and the effects of replacement therapy. We used the SERM (Ralox-A; LY-117018-Hcl) to measure its effects on body physiology in established OVX mice and we followed the differentiation of bone marrow cells. We compared the three experimental groups to determine the overall health of the animals. Protein and ion(s) (Ca, Mg, and Pi) measurement did not reveal an abnormal metabolic activity. Analysis of blood chemistry of rat OVX treated with raloxifene also showed similar metabolic parameters [Black et al., 1994]. In addition, we followed the serum lipid parameters (cholesterol, TG, HDL) and an increase of TG was observed that was correlated with the prolongation of time in OVX mice. The TG level in the OVX-Ralox-A-treated mice was reduced to the sham mice level.

Elevated levels of serum ALK-P in OVX-Ralox-A-treated over OVX mice were noted. An additional source for ALK-P may result from kidney or liver however their activity was unchanged based on other serum analyses we performed. Other studies demonstrated a positive effect of Ralox-A on bone, e.g., bone strength and mass in the OVX rat [Turner et al., 1994]. Histomorphometric analysis [Evans et al., 1994] and X-ray absorptiometry were previously used to measure the positive effect of Raloxifene on OVX rats [Sato et al., 1994; Li et al., 1998].

Estrogen depletion from the circulation ultimately leads to changes in bone physiology. The alterations in cellular differentiation and/or activities are specifically related to osteoclast and osteoprogenitor cells. It is important to understand the cellular alterations and changes related to bone turnover that finally causes bone loss. Thus, in the present study we followed the ovarian hormone effects on stromal and hemopoietic cells of the bone marrow.

The osteoprogenitor cells arise from the marrow stromal cells. The proliferation potential of stromal cells from sham, OVX and OVX-Ralox-A treated mice was quantitated using the CFU-F assay in vitro. A decrease in number of CFU-F colonies from OVX mice was noted when compared to sham mice, and were elevated to control levels in the Ralox-A-treated mice. The results suggested that the replacement therapy affected the stromal stem cell reservoir, which is favorable and leads to changes in bone formation. It is also observed in conjunction with the increase in ALK-P activity measured in the serum of OVX-Ralox-A-treated mice. The function of stromal stem cells is probably affected by estrogen levels, a complex and not yet completely understood process. Estrogen has a direct effect on cells that function in the process of endochondreal bone formation and receptors were demonstrated in chondrocytes in rat, mice [Nasatzky et al., 1994; Pinus et al., 1993], or human models [Ben-Hur et al., 1993] and osteoblasts [Eriksen et al., 1988; Komm et al., 1988; Shamay et al., 1996]. A net bone formation was observed when OVX mice and rats were treated with estradiol or raloxifene [Schwartz et al., 1991; Ornoy et al., 1994; Turner et al., 1994; Li et al., 1998; Lane et al., 1999]. The estrogen has also been demonstrated to affect various cellular activities in cultured osteoblasts such as protein synthesis, ALK-P activity [Shamay et al., 1996], and cytoskeleton organization of cells [Benayahu, 1997]. Additionally, the 17β-estrogen has an immediate effect on osteoblasts by affecting the cell communication through gap junction formation [Massas et al., 1998; Schirrmacher and Bingmann, 1998].

Analysis of hemopoietic cell proliferation and differentiation especially of granulocyte/macrophage progenitors, was assayed by formation of GM-CFU colony in selective culture conditions or by FACS. In this study, the mice were OVX for a long period (4.5 to 6 months). Following this period an augmentation in the G/M cells from OVX mice that were lowered following the Ralox-A-treatment was measured. The estrogen loss caused an increase in GM-CFU that upregulated the osteoclastogenesis in mice, in a process believed to be mediated by IL-6 [Manolagas and Jilka, 1995; Jilka et al., 1992, 1995]. These cellular changes provide an understanding that favors the increase of bone resorption when estrogen is depleted.

The effects of sex hormones were shown on lymphopoiesis to act as negative regulators on cell differentiation in culture as well as in vivo. Kincade and colleagues [1994] paid special attention to gonadal hormone effects and lympho-

poiesis augmentation with aging. The knowledge of such regulation is important in order to evaluate the manipulative role of hormones that can cause immunodeficiency during therapy. It was suggested that hormonal changes such as those seen during pregnancy or with estrogen/progesterone treatment in vivo modulate the differentiation of cells. The progenitors of non-lymphoid hemopoietic cells are not regulated in the same way as the lymphopoietic cells [Kincade et al., 1994; Gaunt and Pierce, 1985; Masauzawa et al., 1994]. In this study, we quantitated the frequency of lymphoid B-cells that were unchanged among the three experimental groups. In another mice model, it was shown that shortly after OVX surgery, B-lymphocytes (B-220+ve) were selectively increased, and myeloid cells were decreased or did not change appreciably. When OVX mice were treated with estrogen, the increased B lymphopoiesis returned to normal [Masauzawa et al., 1994]. The differences observed between these mice experiments are due to the length of OVX period. This may be the basis for the differential alternation in B-cell levels, which were elevated after short OVX period [Masauzawa et al., 1994], or no change after long period (described in this study). The observation of differential changes of lymphopoiesis or myeloid cell differentiation seems to be altered by the hormonal state between sham, OVX treated animals. The up-regulation of granulocyte/monocyte cells is altered following prolongation of OVX period in animals. Thus the differences observed in lymphoid/myeloid cell population rely on the difference in the period after OVX. The pattern of regulation between lymphopoiesis and myelopoiesis revealed a different process.

In conclusion, the established OVX mice experimental model enables us to analyze the therapeutic Ralox-A effects on stromal and hemopoietic cells. The results indicate that Ralox-A has the ability to repress the differentiation of myeloid cells believed to be osteoclast precursor cells and to induce differentiation of stromal stem cells including osteoprogenitors. Such treatment has a net beneficial positive effect on bone formation activity in OVX-Ralox-A-treated mice. This study provides valuable information on the differentiation of cells in the bone marrow microenvironment and also, for the first time, special attention was paid to the differentiation of cells of the immune system under Ralox-A treatment. We suggest that this information will finally lead to a better understanding of the cellular changes that occur during osteoporosis and its treatment.

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