Proliferation of Tartrate-Resistant Acid Phosphatase Positive Multinucleate Cells in Ovariectomized Animals (43120)

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Abstract. **In order to explore why ovarian hormone deficiency causes excessive osteoclastic bone resorption that results in osteoporosis in a large number of postmenopausal women, bone marrow cells from ovariectomized and sham-operated female mice were cultured for 8 days. The cells gave rise in culture to tartrate-resistant acid phosphatase-positive multinucleate cells. The formation of these osteoclast-like cells was enhanced by parathyroid hormone and 1,25(OH)$_2$vitamin D$_3$, with the latter being more effective. Cultures of cells from ovariectomized animals formed significantly more tartrate-resistant acid phosphatase-positive multinucleate cells than those from sham-operated controls. These findings support the hypothesis that ovarian hormone deficiency promotes the expansion of a pool of marrow-derived progenitor cells that differentiate into bone-resorbing osteoclasts under the influence of osteotropic hormones.**

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Aging is associated with an increase in bone loss that results in osteoporosis in a significant fraction of the population. Osteoporosis is more common in females than in males, and in 25–30% of postmenopausal women the bone loss results in major orthopedic problems (1). Both human and animal studies indicate that ovarian hormone deficiency causes an increase in bone destruction by osteoclasts (2–5). However, the underlying basis for the excessive osteoclastic bone resorption is not fully understood.

The recent advances that are being made to define the hematopoietic origin of osteoclasts (6–9) may hold the key to our understanding of the relationship between increased osteoclastic bone resorption and ovarian dysfunction. Accumulating data support the notion that osteoclasts derive from mononuclear precursors from the bone marrow. This view is buttressed, in part, by reports that marrow-derived mononuclear cells of the monocyte-macrophage cell lineage give rise in culture to multinucleate osteoclast-like cells that share with true osteoclasts ultrastructural characteristics, the capacity to resorb calcified bone, and appropriate response to osteotropic hormones (10–14). In addition, the marrow-derived osteoclast-like cells contain tartrate-resistant acid phosphatase, the marker enzyme for osteoclasts (10–14). These observations, which have been made in man (10), baboons (11), cats (12), and mice (13, 14), make the bone marrow culture system a potential model for exploring the pathogenesis of human diseases in which osteoclastic bone resorption is increased (11). Recently, such an exploration in a murine model was initiated, and the findings are the subject of this report.

It was hypothesized that ovarian hormone deficiency enhances the expansion of an early pool of marrow-derived progenitor cells that differentiate to osteoclasts under the influence of osteotropic hormones. To test this hypothesis, we carried out four studies using the ovariectomized mouse and the osteotropic hormones, 1,25(OH)$_2$vitamin D and parathyroid hormone.

Materials and Methods

All studies were carried out with 8-week-old female ICR mice from Harlan Sprague-Dawley, Indianapolis, Indiana. The care of animals was in accord with our institutional guidelines. In the first study, the optimum time for sacrificing mice following surgical removal of their ovaries was determined. Mice were ovariectomized or sham operated and sacrificed at weekly intervals for 4 weeks, and their marrow cells harvested and

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cultured. From this preliminary study, it was decided to sacrifice animals 2 weeks after surgery in subsequent experiments.

In the second experiment bone marrow cells were harvested from the femurs and tibias of ovariectomized and sham-operated mice to assess the influence of ovariectomy on the formation of tartrate-resistant acid phosphatase (TRAP)-positive multinucleate cells (MNC) from mononuclear marrow cells in a culture medium containing 10^{-8} *M* 1,25(OH)$_2$vitamin D. The third study was similar to the second, except that the culture medium contained 100 ng of rat parathyroid hormone (rPTH 1-34)/ml instead of 1,25(OH)$_2$vitamin D. The final study was undertaken to inquire whether the formation of TRAP positive multinucleate cells induced by ovariectomy has an absolute requirement for parathyroid hormone and 1,25(OH)$_2$vitamin D. Marrow cells were again harvested from ovariectomized and control mice 2 weeks after surgery and cultured in a medium that did not contain these hormones.

The mouse bone marrow culture system for the formation of osteoclast-like cells from mononuclear precursors (13), which has been characterized and shown to respond in a manner similar to the osteotropic hormones as human bone marrow cells (10, 15), was used in these studies. Briefly, marrow was harvested from the femurs and tibias. Single-cell suspension was prepared and the mononuclear cells counted by means of a hemocytometer. A total of 0.5×10^6 cells in 0.5 ml of culture medium was plated per well in 24-well plastic plates. The cells were cultured for 8 days at 37°C in a humidified atmosphere of 5% CO_2 in air. The culture medium consisted of bicarbonate-buffered α-minimum essential medium containing 10% fetal calf serum. At the termination of the culture, the plates were stained for TRAP-positive cells and counterstained with hematoxylin using a Sigma kit (St. Louis, MO). TRAP-positive cells with three or more nuclei were counted.

Results

The cultures of the bone marrow cells of animals killed 1 week after surgery had the highest number of

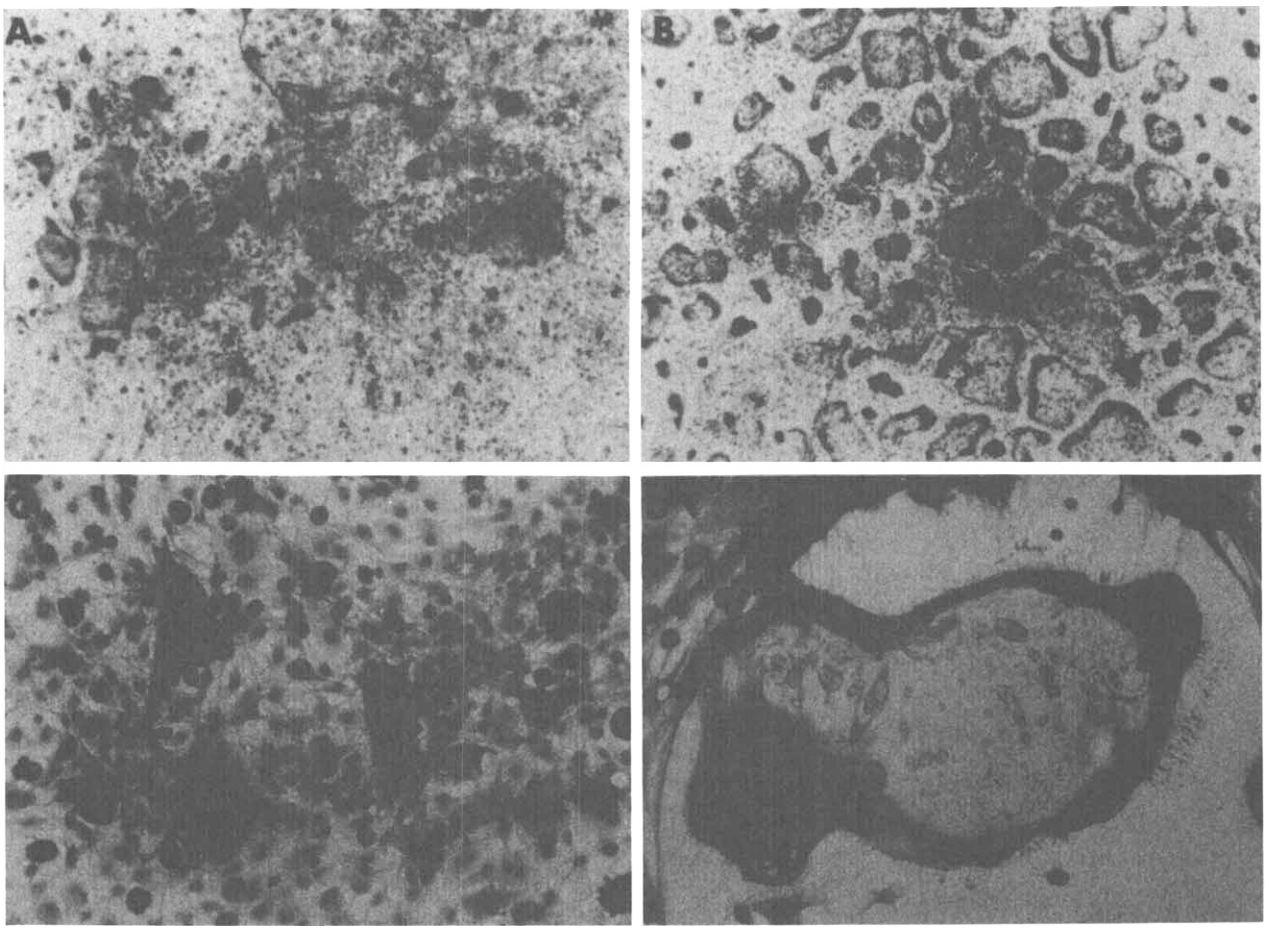

Figure 1. TRAP-positive, MNC formed from mononuclear bone marrow cells from the femurs and tibias of sham-operated (A) and ovariectomized (B) mice (original magnification ×40). The cell nuclei are either located centrally (C) or at the periphery of the cells (D) (original magnification ×200) as has been previously reported (7). The cells with nuclei at the periphery are usually clustered together in colonies as in A and B, whereas those with centrally located nuclei are often scattered randomly in the culture. The marrow cells were cultured as described in the text with a medium containing 10^{-8} *M* 1,25(OH)$_2$vitamin D.

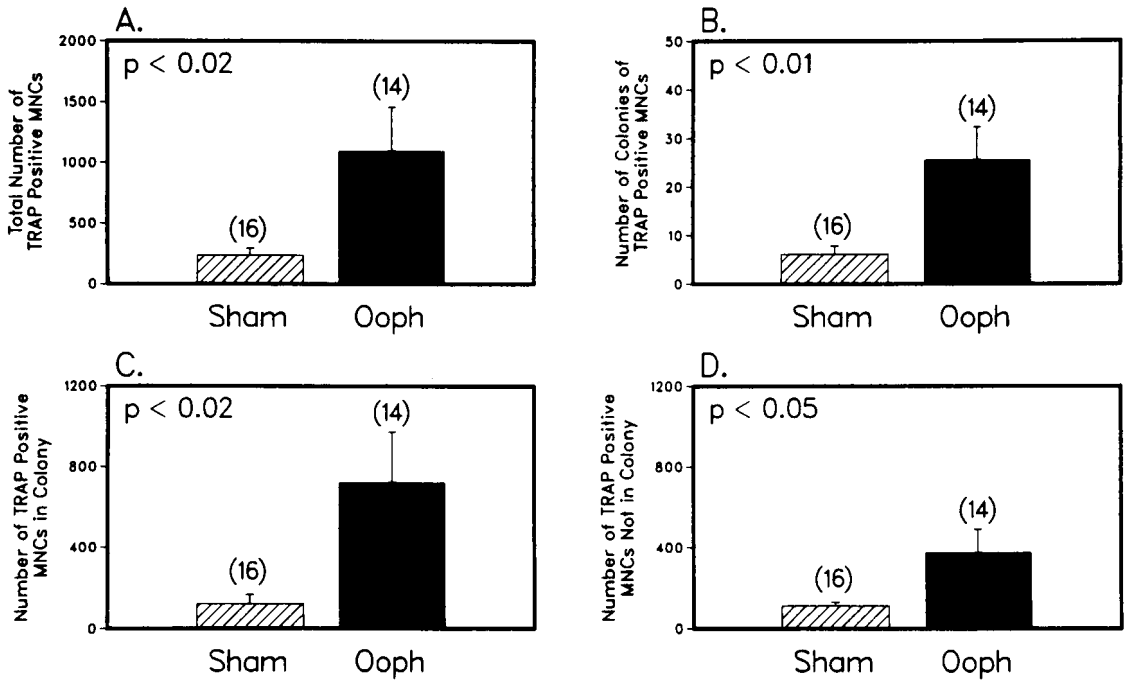

Figure 2. Effect of ovariectomy on the formation of TRAP-positive MNC from mononuclear bone marrow cells in 1,25(OH)$_2$vitamin D-treated cultures. Female ICR mice were ovariectomized (OOPH) or sham operated (SHAM). Two weeks later bone marrows from the femurs and tibias were harvested and cultured as described in the text with a medium containing 10^{-8} *M* 1,25(OH)$_2$vitamin D. TRAP-positive MNC with three or more nuclei were counted. Each bar is mean ± SE. In this and subsequent studies, the difference between means was estimated by Student's *t* test. *P* values denote difference between SHAM and the corresponding OOPH, and $P \leq 0.05$ is considered statistically significant. The numbers in parentheses denote the number of animals per group.

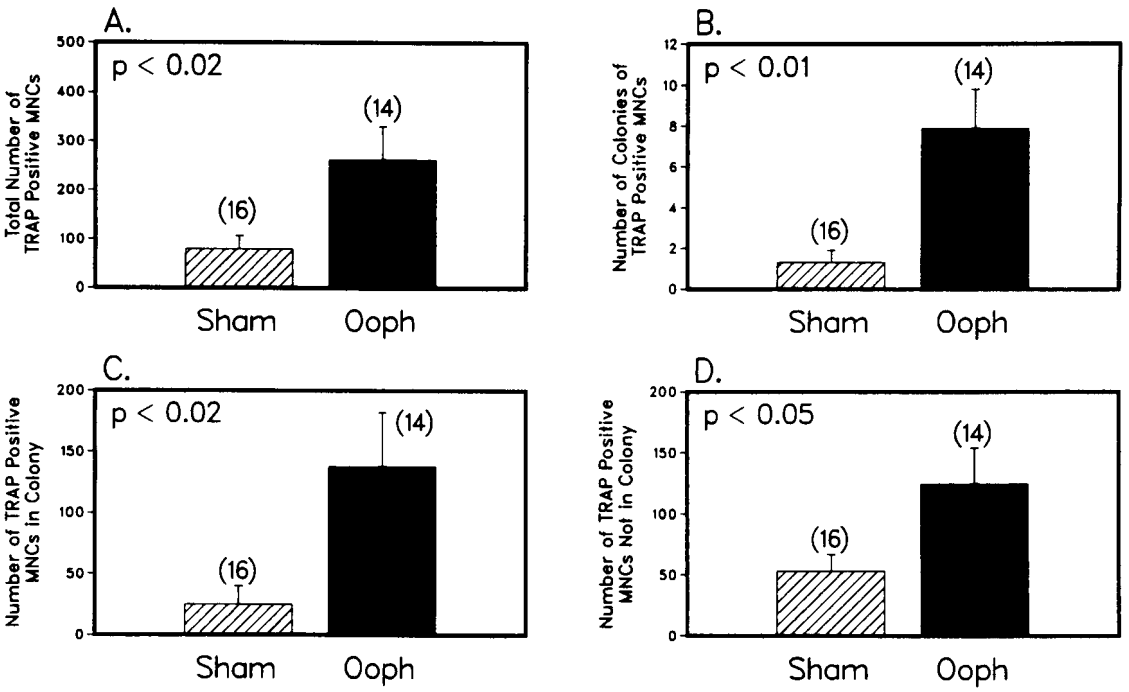

Figure 3. Effect of ovariectomy on the formation of TRAP-positive MNC from mononuclear bone marrow cells in parathyroid hormone-treated cultures. Female ICR mice were ovariectomized (OOPH) or sham operated (SHAM). Two weeks later bone marrows from the femurs and tibias were harvested and cultured with a medium containing 100 ng of rat parathyroid hormone (rPTH 1-34)/ml as described in the text. The TRAP-positive MNC with three or more nuclei were counted. Each bar is mean ± SE. *P* values denote significant difference between SHAM and the corresponding OOPH. The numbers in parentheses denote the number of animals per group.

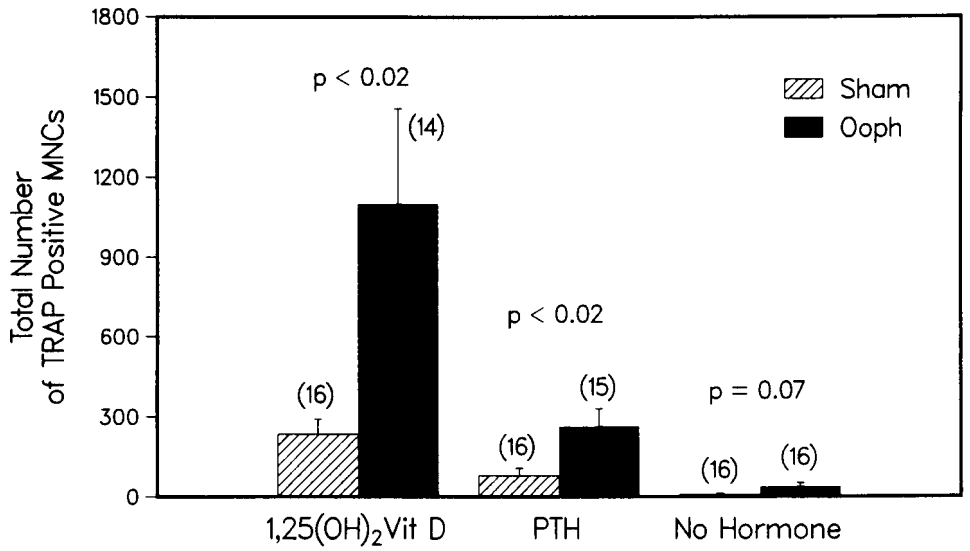

Figure 4. Comparison of the effects of 1,25(OH)$_2$vitamin D, parathyroid hormone (PTH), and no hormone treatment on ovariectomy-induced formation of TRAP-positive MNC. Female ICR mice were ovariectomized (OOPH) or sham operated (SHAM). Two weeks later bone marrows from the femurs and tibias were harvested and cultured without the addition of any hormone (no hormone) as described in the text. TRAP-positive multinucleate cells with three or more nuclei were counted. The data are compared with those from cultures treated with 1,25(OH)$_2$vitamin D or parathyroid hormone (PTH) from Figures 2 and 3. Each bar is mean ± SE. *P* values denote significant difference between SHAM and OOPH in the same treatment groups. $P \leq 0.05$ is considered statistically significant. The numbers in parentheses denote the number of animals per group.

TRAP-positive multinucleate cells. In the following 3 weeks, the number of these cells decreased to a level that did not vary markedly between the different weeks (data not shown). It was these preliminary findings that led the author to terminate subsequent experiments 2 weeks following ovariectomy.

The second study was designed to assess the influence of ovariectomy on the formation of TRAP-positive multinucleate cells from mononuclear marrow cells in a culture medium containing 10^{-8} *M* 1,25(OH)$_2$vitamin D. At the termination of the culture, it was observed that some of the TRAP-positive multinucleate cells were aggregated in circumscribed areas or colonies while the others were scattered randomly in the culture (Fig. 1). The colonies contained 66% of the total number of TRAP-positive multinucleate cells in cultures derived from ovariectomized animals and 50% in the cultures derived from sham-operated controls. The effect of ovarian hormone deficiency on the number of TRAP-positive multinucleate cells was unequivocal. Compared with the sham-operated controls, there was a 4.6-fold increase in the number of TRAP-positive multinucleate cells in the cultures derived from marrow cells from ovariectomized animals ($P < 0.02$) (Fig. 2A). The number of colonies, the number of TRAP-positive multinucleate cells in the colonies, and the number of these cells outside the colonies were also significantly higher in the ovariectomized animals than in controls (Fig. 2B–D).

In the third study the effect of ovariectomy on the proliferation of TRAP-positive multinucleate cells from mononuclear bone marrow cells in the presence of parathyroid hormone was examined. The findings were qualitatively similar to those observed in the cultures treated with 1,25(OH)$_2$vitamin D. The total number of TRAP-positive multinucleate cells, the number of colonies of these cells, the number of TRAP-positive multinucleate cells within the colonies, and the number not in colony were all significantly higher in cultures from ovariectomized than control animals (Fig. 3). However, there was a quantitative difference between the responses to 1,25(OH)$_2$vitamin D and parathyroid hormone with the 1,25(OH)$_2$vitamin D-treated cultures containing 3- to 4-fold more TRAP-positive multinucleate cells.

In the final study, a remarkably depressed proliferation of TRAP-positive multinucleate cells in the absence of parathyroid hormone and 1,25(OH)$_2$vitamin D was observed. Notwithstanding, the number of TRAP-positive multinucleate cells was slightly higher in the cultures from ovariectomized animals, although not significantly ($P = 0.07$, Fig. 4).

Discussion

The findings from these studies indicate that cultures of marrow cells from ovariectomized rats form significantly more TRAP-positive multinucleate cells (MNCs) than those from sham-operated controls. The doses of the osteotropic hormones used in these studies are high compared with their circulating levels in blood. Although lower doses are also effective, the higher doses the author used were found by other investigators to be

optimum for promoting the formation of TRAP-positive multinucleate cells in cultures of mononuclear marrow cells derived from mice (13, 14). The author's findings that $1,25(OH)_2$vitamin D was much more effective than parathyroid hormone in stimulating the formation of osteoclast-like cells and the markedly decreased proliferation of these cells in the absence of the osteotropic hormones are in line with previous findings (13, 14).

The mechanism by which osteotropic hormones increase the formation of TRAP-positive osteoclast-like cells from progenitor cells is uncertain. It has been proposed that these hormones induce the differentiation of early pre-osteoclasts to late pre-osteoclasts and promote the fusion of the latter to form multinucleate osteoclast-like cells (9). It is also uncertain how the lack of ovarian hormones mediate the effects we observed in this study. One interpretation of these findings is that ovarian hormones, directly or indirectly, suppress the formation of osteoclast progenitors. Consequently, ovarian hormone deficiency due to ovariectomy removes this inhibitory influence and thereby enhances the proliferation of an early pool of the progenitor cells. In the presence of $1,25(OH)_2$vitamin D or parathyroid hormone, these progenitor cells are then converted to osteoclasts. Therefore, while a change in the circulating levels of the osteotropic hormones used in this study is not required for the bone loss that results from ovarian hormone deficiency (5, 16), our current findings indicate that the presence of these hormones would be necessary for the optimum proliferation of the bone-resorbing cells necessary for ovarian hormone deficiency bone loss to occur. Although this interpretation of our findings is plausible, it should be recognized that there is some controversy as to whether TRAP-positive multinucleate cells formed from marrow-derived mononuclear cells are authentic osteoclasts (17). Nevertheless, the marked increase in the proliferation of these osteoclast-like cells in ovariectomized animals support the author's hypothesis that an increase in the early pool of osteoclast precursors is an etiologic component of the increased osteoclastic bone destruction that occurs in ovarian hormone deficiency bone loss. These findings indicate that this hypothesis warrants further exploration.

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