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Bone Blood Flow and *In Vitro* Proliferation of Bone Marrow and Trabecular Bone Osteoblast-Like Cells in Ovariectomized Rats

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Summary. Ovariectomy in the rat induces a rapid osteopenia associated with an elevated bone turnover. One hundred and twenty-day-old rats were ovariectomized (OVX) or sham-operated (n = 6–8 per group and per time period studied). ^{45}Ca accretion rate and bone blood flow (microspheres trapping technique) in the femurs were determined at 28, 42, 84, and 119 days after ovariectomy. Both parameters were markedly increased by 84 days and subsided thereafter. At the 42nd day, when bone turnover was maximal, bone marrow and trabecular bone cultures were obtained from sham-operated and ovariectomized animals (n = 10/group). Proliferation rate of bone marrow cells and trabecular osteoblast-like cells estimated by fibroblast colony-forming units (FCFU) efficiency and cell counting was markedly increased in primary and secondary cultures in ovariectomy. These data fitted well with the enhanced number of osteoblasts observed *in situ* in the long bone metaphyses of estrogen-depleted animals. As estrogens were shown in the literature to inhibit proliferation of the red cell line and of other hemopoietic lines, it is possible that estrogens, through a general mechanism, inhibit hemopoietic and stromal lines and also the proliferation of bone marrow-derived trabecular bone cells.

Key words: Rat – Osteoblast-like culture – Ovariectomy – Estrogens – Bone blood supply.

Ovariectomy induces in the rat a rapid osteopenia associated with an elevated bone turnover [1–5], and is thus often used as a model for postmenopausal osteoporosis. The initial rapid phase of bone loss (up to 3 months postovariectomy) is followed by a relative stabilization of bone volume, but at 9 months postovariectomy, a slow phase of bone loss still occurs, again associated with an increased bone turnover [6, 7]. The initial and late phases of bone loss were characterized by markedly increased osteoblastic surfaces [1, 2, 6, 7], increased numbers of osteoblasts per cross section [4], and increased mineralizing surfaces [1, 2, 4–7]. During the subsiding phase of bone loss extending from 3 to 9 months postoperatively, bone formation rate and mineralizing surfaces declined to control levels whereas osteoblast surfaces remained increased compared with controls [6, 7].

In beagle dogs 4 months after ovariectomy, Malluche et al. [8] also described an increased number of osteoblasts, but in this model, bone formation rate per cell was decreased, as was the mineral apposition rate. In spite of the high bone cell density observed in ovariectomized rats, clonal growth of marrow stromal cells was significantly reduced 2–6 months postovariectomy, as shown by Tabuchi et al. [9].

In the present work, two parameters of bone tissue activity were followed over a period of 120 days postovariectomy: the ^{45}Ca accretion rate measuring the rate of calcium apposition and slow exchange [10], and the bone blood flow that has been found to be increased in two rat models of rapid osteopenia (i.e., paraplegia [11] and orchidectomy [12]). At day 42 postovariectomy, which corresponded to the peak of bone tissue turnover, we investigated the *in vitro* proliferation capacity of bone marrow and trabecular bone cells. The results were in accordance with the high number of osteoblasts observed *in vivo*, but there was a discrepancy with Tabuchi's data [9]. On the other hand, they fitted well with published data on the inhibitory effect of estrogens on bone marrow stem cells [13–17].

Materials and Methods

Female Sprague Dawley rats aged 120 days were used. Rats were either ovariectomized using a dorsal approach or sham-operated under ether anesthesia. Food and tap water were available *ad libitum*.

First Experiment

On days 28, 42, 84, and 119 after ovariectomy or sham-operation (n = 6–8/group), length of femurs, bone blood flow, ^{45}Ca accretion rate, and stable calcium content in femurs were determined.

Bone Blood Flow. As previously described [11, 12], 200,000 microspheres (carbonized ^{85}Sr-labeled microspheres 15 μM diameter) (3M Company) in a small volume of saline were injected into the left ventricle shortly before death using a polyethylene catheter introduced through the right carotid artery under nembutal anesthesia. Tail arterial blood was withdrawn at a constant rate ("V") for 70 s, beginning 10 s before the injection. The animals were killed by intracardiac injection of potassium chloride. Femurs were separated from the soft tissues and were then cut into four pieces (distal epiphysis, distal metaphysis, bone shaft, and proximal end of bone). ^{85}Sr radioactivity was measured in the blood sample ("a") and in the bone pieces ("b"). Bone blood flow (BBF) was calculated as:

$$BBF(\mu l/min) = V(\mu l/min) \times b/a$$

Bone Accretion Rate. Fifty microcuries of ^{45}Ca chloride (IRE, Belgium) was injected i.p. 72 hours before death. Plasmatic specific activity was determined in blood obtained from the tail at 6, 24, and 72 hours. The bone fragments, already used for bone blood flow

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measurements, were ashed and dissolved in 12 N HCl to count ^{45}Ca in a liquid scintillation spectrometer (Packard Tricarb). Stable calcium was measured by atomic absorption spectrometry. The Ca accretion rate (mg/24 hours) was calculated for each bone fragment by multiplying the average bone retention of ^{45}Ca by the mean of the plasmatic specific activity.

Length of femur was measured with a caliper.

Second Experiment

At 42 days after surgery, sham-operated and ovariectomized animals (n = 10/group) were killed by decapitation. Serum parameters measured were Ca, P, follicle-stimulating hormone (FSH) (reagents kindly provided by the NIDDK), alkaline phosphatase (Boehringer Mannheim), osteocalcin (Biomedical Technologies Inc.).

Morphometric Measurements. Tibial metaphyses of sham-operated and ovariectomized animals were fixed in acetone and embedded undecalcified in methyl methacrylate/glycol methacrylate 2:1 (v/v). Longitudinal sections of 5–7 μm thickness were cut with a Reichert Jung 1140 Autocut microtome and stained for tartrate-resistant acid phosphatase activity (osteoclast counting) or with modified hematoxylin-eosin (osteoblast counting). The number of osteoblasts and osteoclasts was determined along the metaphyseal part of the growth plate in an area 0.02 mm wide by using a grid with an analyzed surface of 0.072 mm^2 under a 500× magnification (15 fields of 0.072 mm^2 were examined for each animal). The results were expressed in number of cells/mm^2 of analyzed surface.

Both femurs of each animal (n = 7/group) were used for cell cultures.

Bone Marrow Culture

Bone marrows were flushed from the midshafts of the femurs with 3 ml medium. Cells were dispersed by repeated aspirations through a 21-gauge needle, and counted in a hemocytometer. Cell counting performed on 50 pairs of femurs indicated an intra-animal coefficient of variation of 4%. Viability of the cells was assessed using the trypan blue exclusion method.

Primary Culture. Viable cells (2×10^7), including red blood cells, were plated in 25 cm^2 tissue culture flasks (Nunc Products) in phenol red-free Minimum Essential Medium (MEM) (Gibco) supplemented with 15% fetal calf serum (FCS) (Gibco), 50 μg/ml ascorbic acid (Gibco), 2 mM L-glutamine (Gibco), 50 μg/ml gentamycine, and 2.5 μg/ml fungizone. Medium was changed 48 hours after inoculation and then every third day. Cells were incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2.

Eight days after inoculation, tissue culture flasks (n = 3/animal) were fixed in 10% formaldehyde (Merck) in phosphate-buffered saline (PBS) and stained for alkaline phosphatase (Sigma, kit 85 L-2). The numbers of FCFU containing at least 50 cells were counted. Ten days after plating, primary cultures (n = 3/animal) were stopped and adherent cells were trypsinized (trypsin 0.5 g/liter—EDTA 0.2 g/liter) and counted in a hemocytometer.

Secondary Cultures. The harvested cells were seeded at a density of 5×10^3 cells/cm^2 (n = 3/animal) in 10 cm^2 tissue culture wells. Eight days later, the cells were trypsinized and counted again in a hemocytometer.

Trabecular Bone Culture

Osteoblast-like cells were obtained by the explant technique: the whole trabecular content was scraped with a scalpel from each femoral metaphysis and was extensively washed with PBS to remove blood and marrow elements.

Primary Culture. The trabecular fragments were seeded in 75 cm^2 tissue culture dishes (Nunc Products) in phenol red-free MEM supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 50 μg/ml gentamycine, and 2.5 μg/ml fungizone (4 dishes/animal). The medium was changed twice a week. After 4 weeks of culture, the cells were released using trypsin—EDTA solution (0.5 and 0.2 g/liter, respectively). The cells obtained from the four plates of each animal were pooled and counted in a hemocytometer.

Secondary Culture. The harvested cells were seeded at a density of 5×10^3 cells/cm^2 (n = 3/animal) in 10 cm^2 tissue culture wells. Eight days later the cells were trypsinized and counted.

Characterization of Cells

As previously described [18], the cells were characterized by an intense alkaline phosphatase staining, by their sensitivity to osteotropic hormones, and by osteocalcin production in response to $1,25(OH)_2D_3$ 10^{-8} M.

Pools of bone marrow and trabecular bone cells from sham-operated and ovariectomized animals were constituted after first trypsinization, and were used to study two phenotypic characteristics of osteoblast-like cells, i.e., alkaline phosphatase production in response to $1,25(OH)_2D_3$ and cAMP production in response to parathyroid hormone.

Alkaline Phosphatase Response to $1,25(OH)_2D_3$. The cells were plated at a density of 5×10^3 cells/cm^2 in MEM + 2% FCS for 48 hours and then incubated in MEM + 0.1% bovine serum albumin (BSA) and stimulated with 10^{-10}–10^{-8} M $1,25(OH)_2D_3$ (a gift from Roche-Brussels) for 48 hours. Vehicle medium of the hormone was added in control cultures. At the end of the incubation period, the culture medium was removed. The cell layer was washed with PBS and then scraped off the dishes with a rubber policeman and sonicated in distilled water. After centrifugation, the supernatants were used for the determination of alkaline phosphatase activity (Kit CBR Boehringer Mannheim) and of the protein content (method of the molybdate-pyrogallol red, Sopachem). Alkaline phosphatase was expressed in nmoles p-nitrophenol/mg protein/minute.

cAMP Production in Response to PTH. The cells were plated in MEM + 10% FCS and grown for 8 days. They were incubated in MEM + 1% FCS and 5×10^{-4} M isobutyl methylxanthine (IBMX) (Sigma) for 30 minutes at 37°C and then stimulated with 10^{-10} – 10^{-8} M bovine PTH (fragment 1-34) (Sigma) for 5 minutes. At the end of the incubation, the medium was removed and the cells were washed with cold PBS. One milliliter propanol 90% was added and the culture plates were put on ice. After 1 hour, propanol was collected and evaporated. The extracted cAMP was measured with a kit provided by Amersham. cAMP levels were expressed in pmol/10^5 cells.

Statistical Analysis. Comparison between the groups was made with the Student's *t* test and with the nonparametric Wilcoxon test.

Results

First Experiment

Ovariectomy induced a slight increase in bone length, maximal at 42 days postovariectomy but still significant ($p < 0.01$) at 119 days. Calcium content of whole bone (femur) was decreased throughout the study. Bone loss was restricted to the metaphysis whereas diaphyseal calcium content remained similar in sham-operated and ovariectomized rats. ^{45}Ca accretion rate and BBF were increased equally in each piece of the femurs from 28 to 84 days postovariectomy, and then decreased to control values. Figure 1 illustrates the ^{45}Ca accretion rate and the BBF in the whole femur.

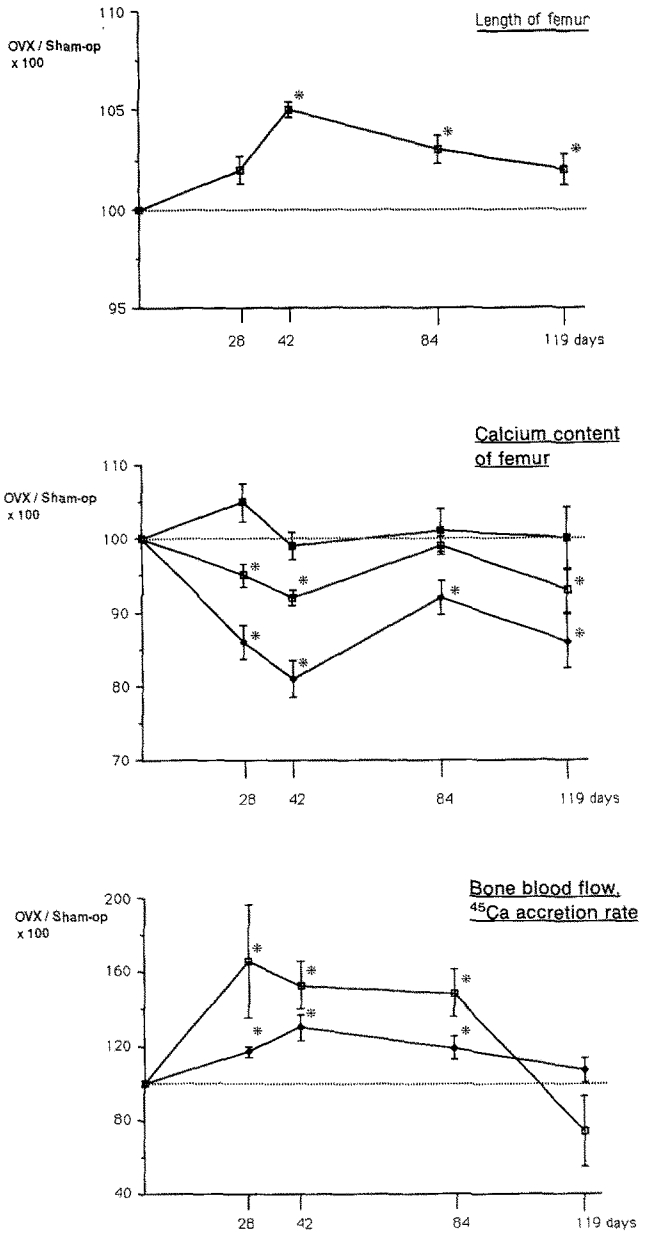

Fig. 1. Evolution of length of femur, calcium content, ^{45}Ca accretion rate, and BBF over time after ovariectomy in 120-day-old rats. Data are expressed in percentage of mean values of sham-operated animals. *A significant difference with sham-operated animals, $P < 0.05$. Nominal values in sham-operated animals at day 0 (n = 7), mean ± SEM. Top graph: length of femur 35.2 ± 0.3 mm; middle graph: calcium content (mg) of whole femur (□) 123 ± 2, lower metaphysis (♦) 21.2 ± 0.4, and diaphysis (■) 45.7 ± 1.7; lower graph: bone blood flow (μl/min) in whole femur (□) 68 ± 8 and ^{45}Ca accretion rate (mg/24 hour) in whole femur (♦) 2.03 ± 0.10.

Second Experiment

Biology at day 42 after ovariectomy (Table 1) shows that the body weight of the ovariectomized animals was significantly higher when compared with sham-operated animals. Ovariectomy led to an eightfold increase in the FSH serum levels. No difference in Ca or P serum levels was found between the groups. Alkaline phosphatase and osteocalcin levels were significantly elevated after ovariectomy. A larger number of cells was obtained from the flushed bone marrow of ovariectomized rats. Differential cell counts from marrow smears failed to show significant differences in cell subpopulations between the groups.

Table 1. Biology at day 42

	Units	n	Sham-operated	Ovariectomized
Weight	g	10	307 ± 3	345 ± 6[a]
Serum				
FSH	ng/ml	10	8.6 ± 0.9	63.3 ± 3.2[a]
Calcium	mg/dl	10	11.7 ± 0.2	11.4 ± 0.2
Phosphorus	mg/dl	10	5.0 ± 0.2	5.4 ± 0.3
Alkaline ph	IU	10	53 ± 4	71 ± 5[b]
Osteocalcin	ng/ml	10	60 ± 2	84 ± 4[b]
Femur				
Nb bone marrow cells	10^8	7	2.2 ± 0.1	2.7 ± 0.1[b]
Tibia				
Osteoblast density	/mm²	8	464 ± 51	632 ± 47[b]
Osteoclast density	/mm²	5	34 ± 5	46 ± 4

The results are expressed in mean ± SEM
Difference with sham-operated animals: [a]$P < 0.001$, [b]$P < 0.05$

Osteoblast density had significantly increased by a factor of 1.5× whereas osteoclast density, though higher in the OVX group, was not significantly different from the control group.

Cell cultures and proliferation rate are shown in Figure 2.

Bone Marrow

The number of FCFU formed at day 8 after inoculation was higher in the cultures from ovariectomized rats when compared with controls: 104 ± 21 and 53 ± 10, respectively; mean ± SEM, $P < 0.05$.

The total number of adherent cells harvested by trypsinization (10 days) was also markedly increased in ovariectomized rats: 4.5 ± 1.7 10^5 cells/flask in OVX compared with 2.3 ± 0.8 10^5 cells/flask in sham-operated, $P < 0.05$.

The rate of proliferation of the cells isolated from OVX rats remained increased after trypsinization and replating (secondary culture): 1.6 ± 0.2 10^5 cells/10 cm² well in OVX rats compared with 1.1 ± 0.1 10^5 cells/10 cm² well in sham-operated, $P < 0.05$.

Trabecular Bone

Initial development of cultures from trabecular explants was faster in the cultures from OVX animals. Twenty-eight days after initiation of the cultures, the mean number of cells harvested per animal was 3.2 ± 0.5 10^6 cells in OVX animals compared with 1.6 ± 0.4 10^6 in sham-operated ones, $P < 0.05$. The proliferation rate of the cells isolated from OVX rats remained higher than controls after trypsinization: 1.4 ± 0.1 10^5 cells/10 cm² well in OVX compared with 1.1 ± 0.1 10^5 cells/10 cm² well, $P < 0.05$.

Phenotypic characteristics are shown in Figure 3.

Bone marrow and trabecular bone cells of ovariectomized and sham-operated animals presented similar response curves to $1,25(OH)_2D_3$ and to PTH. Significant responses were observed for the same hormonal concentrations, 10^{-9} and 10^{-8} M PTH and $1,25(OH)_2D_3$, in both types

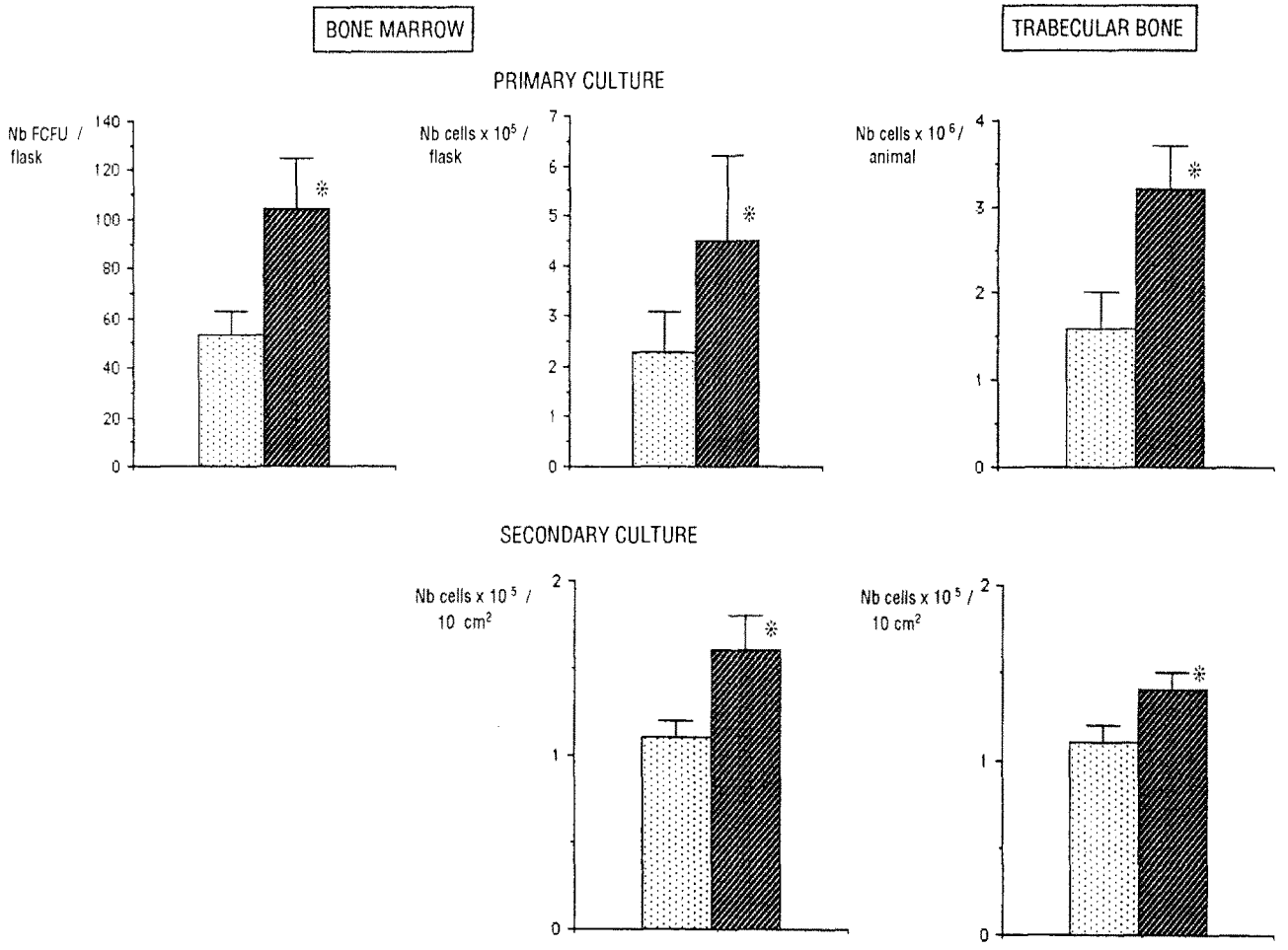

Fig. 2. Proliferation rate of bone marrow and trabecular bone cells in primary and secondary cultures. Sham-operated (▨); ovariectomized (▨). *A significant difference with the cells isolated from sham-operated animals. Each bar is the mean ± SEM of 7 animals.

of cultures. The trend observed for a lower amplitude of response to hormones in OVX rats did not reach statistical significance.

Discussion

The present study confirms and extends several observations made by others on the ovariectomy effects on bone morphology and function in the rat [1–5]. Enhancements in ^{45}Ca accretion rate, metaphyseal osteoblast density, and values of bone formation parameters like alkaline phosphatase and osteocalcin reflect a state of increased bone turnover. The latter is associated with the rapid onset of the osteopenic state evidenced within 4 weeks after ovariectomy. These already-described effects [2–5] occur with a strong increase in BBF that represents a new feature not mentioned in former works. This high BBF is maintained at least from day 28 until day 84 after ovariectomy. Next it decreases, reaching normal values at day 119, i.e., at a moment when ^{45}Ca accretion rate has also subsided. As for the first weeks after oophorectomy there is a parallel increase in BBF, bone turnover, and bone loss occurs; the question arises as to whether these three bone parameters are correlated.

A parallel increase in BBF and bone loss has been noted in paraplegia [11] and orchidectomy [12], two conditions associated with an increased osteoclastic resorption, but with a less significant increase in bone turnover. Bone loss induced in diabetes mellitus [19] is characterized by decreased bone turnover with decreased BBF. However, calcium deprivation in the rat induces a rapid and drastic demineralization with a marked increase in bone turnover, but without any change in BBF (unpublished data). Thus, it turns out that BBF and bone cell activities cannot be easily correlated.

At the 42nd day postovariectomy, we observed an increased number of bone marrow cells in the medullary cavity which is enlarged [3, 4, 20]. Primary cultures of bone marrow show a markedly (2×) increased clonal growth of stromal cells and produce, like the trabecular bone cultures, a larger number of adherent cells with phenotypic characteristics of osteoblasts, indicating a stimulation of osteoprogenitor cells after ovariectomy. The osteoblast-like cells obtained from cultured bone marrow or trabecular bone showed similar response curves to $1,25(OH)_2D_3$ or PTH stimulation. Sensitivity to these hormones was not modified after ovariectomy.

The higher proliferation rate is maintained in secondary cultures for both types of cultures (i.e., after 18 or 35 days of culture for bone marrow or trabecular bone cells, respectively). These results are in agreement with the increase in metaphyseal osteoblasts observed *in situ* by many authors [1, 2, 4, 5] and by us, but at variance with the data of Tabuchi

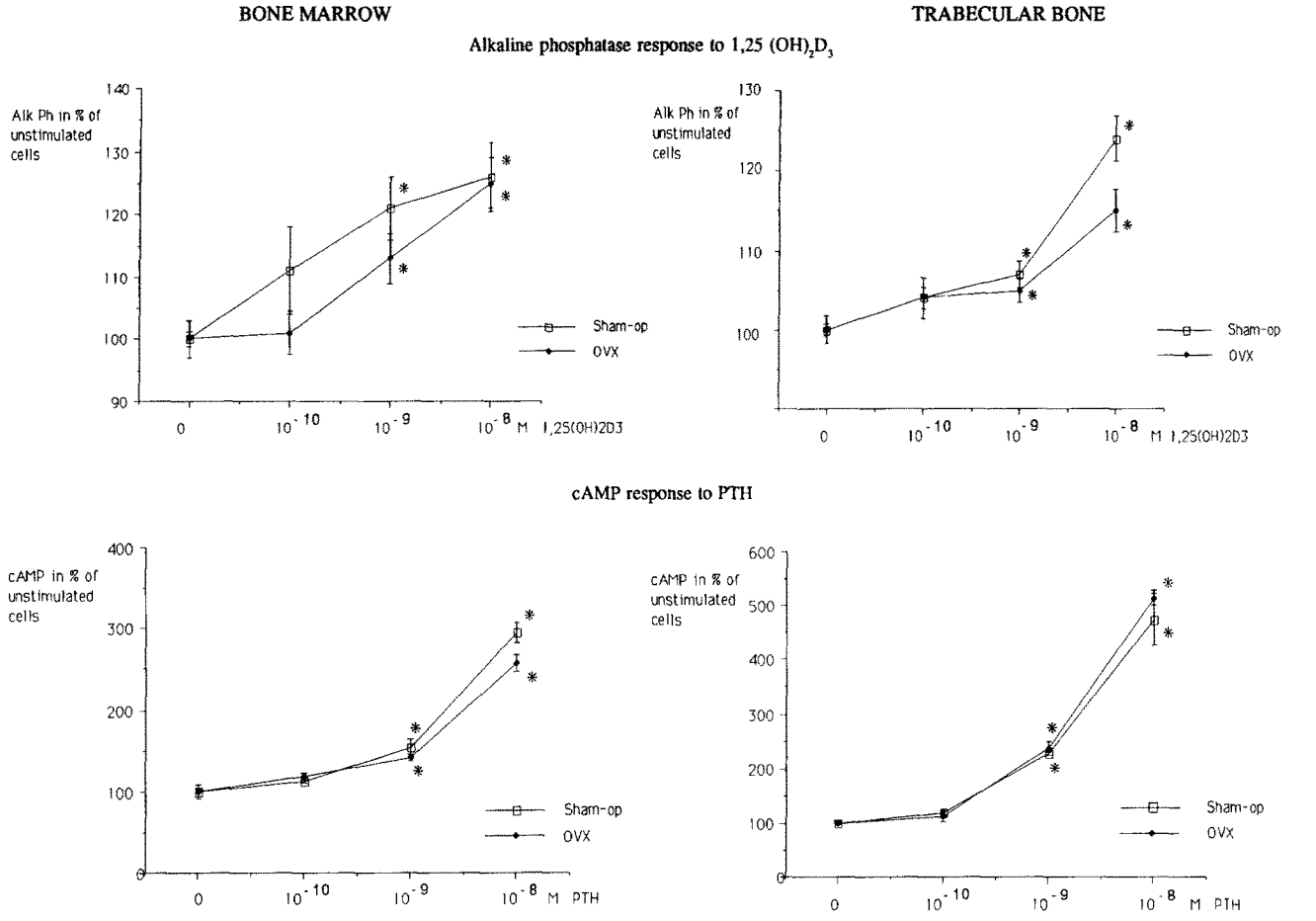

Fig. 3. Response to $1,25(OH)_2D_3$ and PTH of secondary bone marrow and trabecular bone cell cultures from ovariectomized and sham-operated rats. Data are expressed in percentage of unstimulated cultures, mean ± SEM, n = 4. *A significant difference with unstimulated cultures, $P < 0.05$. Significant responses were observed for the same hormonal concentrations (10^{-9} and 10^{-8} M $1,25(OH)_2D_3$, 10^{-9} and 10^{-8} M PTH) in cultures from OVX and sham-operated animals.

et al. [9] who described a decreased proliferative potential of marrow stromal cells 2–6 months after ovariectomy in 50-day-old rats. This divergence could be attributed to differences in temporal sampling of the experiments. Indeed, the observations of Wronski et al. [6, 7] suggest a period of subsiding bone turnover and bone loss between 100 and 270 days following the ovariectomy of 90-day-old rats. During this period, the trend for an increased osteoblastic surface in OVX rats was maintained, but without statistical significance [6]. This phase could be related to the lessening of the hemodynamic (blood flow) and metabolic (^{45}Ca accretion rate) response observed by us 120 days postovariectomy, but also to the deficit in the proliferative capacity of osteoprogenitor cells observed somewhat earlier in younger rats by Tabuchi et al. [9].

Ovariectomy cannot be equated with the sole estrogen depletion. Progesterone for example, has been investigated recently with regard to bone metabolism: bone cells have receptors for this hormone [21, 22] which prevents osteopenia in ovariectomized rats [23] whereas it antagonizes estrogen effect on bone in ovariectomized [23] and intact rats [24]. However, we should like to comment on similarities of estrogen action on hemopoietic and stromal cell lines, with possible relevance to ovariectomy. Estrogens have a suppressive effect on all hemopoietic cell lines [13–17], which is better documented for the red cell line [13–15]. On the other hand, we have shown on cultured rat osteoblast-like cells that 17β-estradiol reduces thymidine incorporation at physiological doses [18], an observation confirmed by Gray et al. [25] but infirmed by Ernst et al. [26]. Systemic administration of estrogens has been shown to reduce bone formation and resorption indices in rats [20, 27, 28]. The recent finding of Takano-Yamamoto and Rodan [29] indicating that direct *in situ* administration of estrogens to OVX rats still increases the osteoblast number while bone mass is restored and the osteoclast number decreases, could possibly be related to the differential effect of physiological and pharmacological doses of estrogens [30, 31].

Notwithstanding the difficulties raised by some published data, a possibility exists that part of the role for estrogens in bone goes through a general inhibition of bone marrow hemopoietic and stromal cell lines proliferation, extended to osteoblasts of the trabecular bone.

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