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OSTEOBLAST-OSTEOCLAST RELATIONSHIPS IN BONE RESORPTION: OSTEOBLASTS ENHANCE OSTEOCLAST ACTIVITY IN A SERUM-FREE CO-CULTURE SYSTEM

Anna Teti[1*], Maria Grano[2,4], Silvia Colucci[2], Francesco P. Cantatore[3], Maria C. Loperfido[3], and Alberta Zambonin Zallone[2]

[1]Institute of Human Anatomy, School of Pharmacy, University of Bari, Policlinico, piazza G. Cesare, 70124 Bari, Italy

[2]Institute of Human Anatomy, School of Medicine, University of Bari, Italy

[3]Cattedra di Reumatologia, University of Bari, Italy

[4]Centro di Citomorfologia Normale e Patologica, University of Chieti, Italy

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Osteoblast-osteoclast relationships in bone resorption are unclear. We investigated whether osteoblasts constitutively influence osteoclast activity. We employed a serum-free co-culture system in which chicken osteoclasts and chick calvaria or, alternatively, isolated chick osteoblasts were cultured in two different compartments separated by a 0.45 μm porous membrane permeable to soluble molecules. Osteoclastic bone resorption, evaluated by release of ^{3}H-proline from prelabeled bone fragments, was significantly enhanced by bone cells resident in the calvaria, as well as by isolated osteoblasts. Stimulation was specific, since periosteal cells, or skin fibroblasts, failed to mimic osteoblast activity. Conditioned medium from osteoblast cultures stimulated osteoclast function in a similar manner, indicating that paracrine signals, capable of crossing the porous membrane separating the two compartments, are released by the bone forming cells. © 1991 Academic Press, Inc.

The mechanisms regulating osteoclast bone resorption have not yet been fully elucidated (1, 2). A substantial body of evidence indicates that osteoclasts are directly regulated by calcitonin (3), retinol (4), low pH (5, 6) and elevated extracellular calcium concentration (7-9), whereas several calciotropic factors, known to stimulate bone resorption *in vivo*, display an indirect effect, via an intermediate cell type. As suggested by Rodan and Martin (10), and indicated by several reports (11-16), the key intermediate target cell for such indirect osteoclast-stimulating factors may be represented by the osteoblast. In this report, we investigated whether osteoblasts were capable of constitutively enhancing osteoclast bone resorption. To this purpose, we employed a co-culture system in which periosteum-free newborn chick calvarial fragments, carrying their *in situ* bone cells, or, alternatively, isolated osteoblasts at the first passage, were co-cultured, in serum-free medium, together with chicken osteoclasts. The compartments in which bone forming and bone resorbing cells were plated, were separated by a 0.45 μm porous membrane, which permitted diffusion of soluble molecules, but prevented cell-cell contact. Results indicated that the bone forming cells, either in their natural bone environment, either isolated, positively affected osteoclast activity, significantly enhancing bone resorption.

*To whom correspondence and reprint requests should be addressed.

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MATERIALS AND METHODS

Cell preparation and culture. Differentiated osteoblasts were obtained from newborn chick calvaria by a modification of a previously described method (18). Briefly, calvaria from 2 day-old chicks were removed, cleaned from soft tissues, and reduced to approximatively 2 mm side fragments. Some fragments, containing bone cells in situ, were immediately used for the experiments (see below). The other fragments were digested with 1 mg/ml Clostridium Histolyticum neutral collagenase in 20mM HEPES-buffered 199 medium for 30 min, at 37°C, then washed (3x) with 199 medium containing 2.2 g/l sodium bicarbonate and supplemented with 20% FCS (FCS-199). Digested fragments were cultured in 10 cm dia. Petri dish (10 pair calvaria/dish) in FCS-199 medium, in a water saturated atmosphere containing 5% CO_2. Osteoblast-like cells, resident in the calvarial explants, proliferated and migrated to the culture substrate, reaching confluence within 3-4 weeks. Cells were then trypsinized and transferred to experimental dishes. All the experiments were performed at the first passage.

Osteoblast phenotype was controlled in semiconfluent monolayers, derived from calvarial fragments, at the first passage. Alkaline phosphatase activity was tested both histochemically, using the Sigma kit (St. Louis, MO), and fluorometrically, using 4-methyl-umbelliferyl-phosphate as substrate. Monolayers were solubilized in 0.1% sodium dodecyl sulphate (SDS). Cell lysate was incubated at pH 10.3 in the presence of 200 µM substrate, at 37°C for 30 min. The 4-methyl-umbelliferone (4-MUMBF) produced by the enzyme was detected monitoring its fluorescence at 369 nm excitation and 448 nm emission wavelenghts, and converted into nmoles/min by standard curves. Results were normalized per mg cell protein, measured according to Lowry et al. (19). cAMP production was examined in semiconfluent monolayers treated with 10^{-8} M PTH in 199 medium supplemented with 1% FCS and 0.1 mM IBMX for 1 hour, by Incstar Corporation Radioimmunoassay (Stillwater, MN). Results were expressed as pmoles of cAMP/mg cell protein. Osteocalcin production was measured by Incstar Corporation radioimmunoassay. Stimulation of ostecalcin synthesis was obtained, as already demonstrated (20), treating the cells with 10^{-8} M 1,25-dihydroxy-vitamin D_3 for 48 hours. Results were expressed as ng osteocalcin/mg cell protein. Collagen synthesis was qualitatively evaluated using polyclonal antibodies raised against type I and type III collagen. Cells were fixed in 3.7% paraformaldehyde for 10 min, followed by 70% ethanol, 5 min, rinsed three times in PBS and reacted, for indirect immunofluorescence, with the rabbit anti-human type I- or mouse anti-human type III collagen for 45 min at 37 °C, followed by the fluorescein-conjugated goat anti-rabbit and sheep anti-mouse IgG, respectively. Morphological analysis was performed by conventional epifluorescence.

Periosteal cells and skin fibroblasts were obtained from endocranial periostea and derma of 2 day-old chicks, respectively. Both tissues were digested with 1 mg/ml neutral collagenase in HEPES-buffered 199 medium for 30 min, at 37°C. Released cells were extensively washed (3x) with FCS-199 medium, plated into 10 cm dia. Petri dishes and incubated in FCS-199 medium, in a water saturated atmosphere containing 5% CO_2. Cells were grown until confluence, then trypsinized and transferred to appropriate dishes for characterization and experiments.

Avian osteoclasts were isolated from laying hen medullary bone, and characterized as already described (21).

Co-culture system. To assess cooperation between bone cells in situ, or, alternatively, isolated osteoblasts, and osteoclasts, a co-culture device (Millipore Products Div., Bedford, MA) was used (Fig. 1). The system

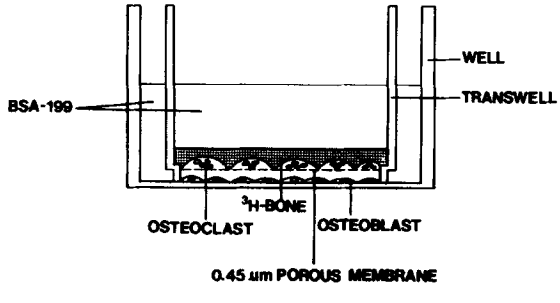

Fig. 1. Scheme representing the co-culture system used for the experiments. In a 16 mm dia. culture well a 12 mm dia. transwell was inserted. The transwell carried at the bottom a porous membrane, pore size 0.45 µm. Osteoblasts, or alternatively, calvarial fragments, periosteal cells or skin fibroblasts, were cultured in the lower compartment. Osteoclasts were plated in the upper compartment in the presence of ^{3}H-proline prelabeled 25-55 µm bone fragments. The two cell types were, therefore, separated by the membrane, which prevented cell-cell contact, but allowed exchange of soluble molecules capable of passing through the 0.45 µm pores.

was formed by 24 well culture plates (16 mm dia.) and by Millicell-HA (12 mm. diametrer) inserted traswells, carrying at the bottom a 0.45 um porous membrane. In each well the porous membrane segregated an upper and a lower compartment suitable for cell culture. Cells plated in the two compartments were prevented from establishing cell-cell contact by the membrane. However, due to the pore diameter (0.45 um), soluble molecules with various molecular weight could diffuse between the two compartments. This device could, therefore, unmask cell-cell cross-talk occurring via secretion of specific paracrine factors, non involving direct cell-cell contact.

Experimental protocol. Osteoblasts at the first passage were plated in 24 well plates and grown to confluence in FCS-199 medium. Osteoclasts were cultured in Millicell transwells and cultured for 24 hours in MEM supplemented with 10% FCS. 24 hour old osteoclast-containing transwells were then transferred into confluent osteoblast-containing wells. Therefore, the upper compartment carried osteoclasts, whereas the lower compartment carried osteoblasts. Wells containing exclusively osteoclasts were used as controls. Furthermore, to assess the cooperation between bone cells in situ and osteoclasts, some experiments were performed incubating in the lower compartment periosteum-free calvarial fragments. Specificity was evaluated substituting osteoblasts with periosteal cells or skin fibroblasts.

To assess bone resorption, media were replaced with 1.5 ml serum-free 199 containing 0.5% BSA (BSA-199). 400 ug/ml 25-55 um dia. ^{3}H-proline in vivo-prelabeled chick bone fragments were added to the osteoclast-containing compartment. After 24 hours, media were replaced with fresh BSA-199 and cells were incubated for further 24 hours. 1 ml media harvested after the first and after the second 24 hour incubations were transferred to vials in the presence of 9 ml scintillation fluid, and radioactivity was measured by a Beckman 6500 B-counter. To demonstrate whether osteoblast effect was due to soluble paracrine factors, osteoclasts were also incubated with 50% freshly harvested BSA-199 medium conditioned, for 24 hours, by confluent osteoblast cultures. Experiments were performed in quadruplicate at least 4 times. Radioactivity was converted to ug bone resorbed according to Blair et al. (17). Results were expressed as mean ug bone resorbed $\pm$ SE. Statistic significance was calculated by the t Student test.

Morphology. Histological controls were performed on freshly prepared calvarial fragments, and on fragments at the end of the experimental period. Fragments were fixed in reduced Karnowsky (22) (1 hour, 4^{o}C), post-fixed in 1% osmium tetroxide in cacodylate buffer (1 hour, 4^{o}C) and processed for Araldite-Durcupan embedding. Semithin sections were cut with a LKB Ultrotome IV and stained with 0.1% toluidine blue. Cell culture morphology was evaluated in phase contrast.

RESULTS

Histological control. Freshly prepared periosteum-free calvarial fragments, observed in semithin sections, showed a number of osteocytes resident in their lacunae as well as endothelial and connective cells laying in the vascular channels. An incomplete layer of flattened osteoblasts were also present, laying on the bone surfaces.

Calvarial fragments cultured for 48 hours presented a layer of osteoblast-like cells (Fig. 2) surrounding the entire bone. Osteocytes, endothelial and connective cells in the vascular channels appeared well preserved and unmodified compared to the freshly prepared fragments.

Osteoblast characterization. Cells migrating from the collagenase digested calvarial fragments were polygonal (Fig. 3), showed slow growth rate, and reached confluence within 3-4 weeks. At the first passage, they retained their morphology, showed high alkaline phosphatase activity, PTH-dependent increase of cAMP, 1,25-dihydroxyvitamin D_3-stimulated osteocalcin synthesis, expression of high level of type I collagen, and very low level of type III collagen (Table1). All these parameters were consistent with an osteoblastic phenotype. Therefore, we will refer to these cells as osteoblasts.

Bone resorption. Control osteoclasts, plated in serum-free 199 medium containing 0.5% BSA in the presence of 400 ug/ml of radioactively labeled bone fragments, showed a basal level of bone resorption ranging from 3.6 $\pm$ 1.2 to 12.8 $\pm$ 1.9 ug bone resorbed by 50,000 cells/1.6 cm well/24 hours according to radioactive bone and cell preparation.

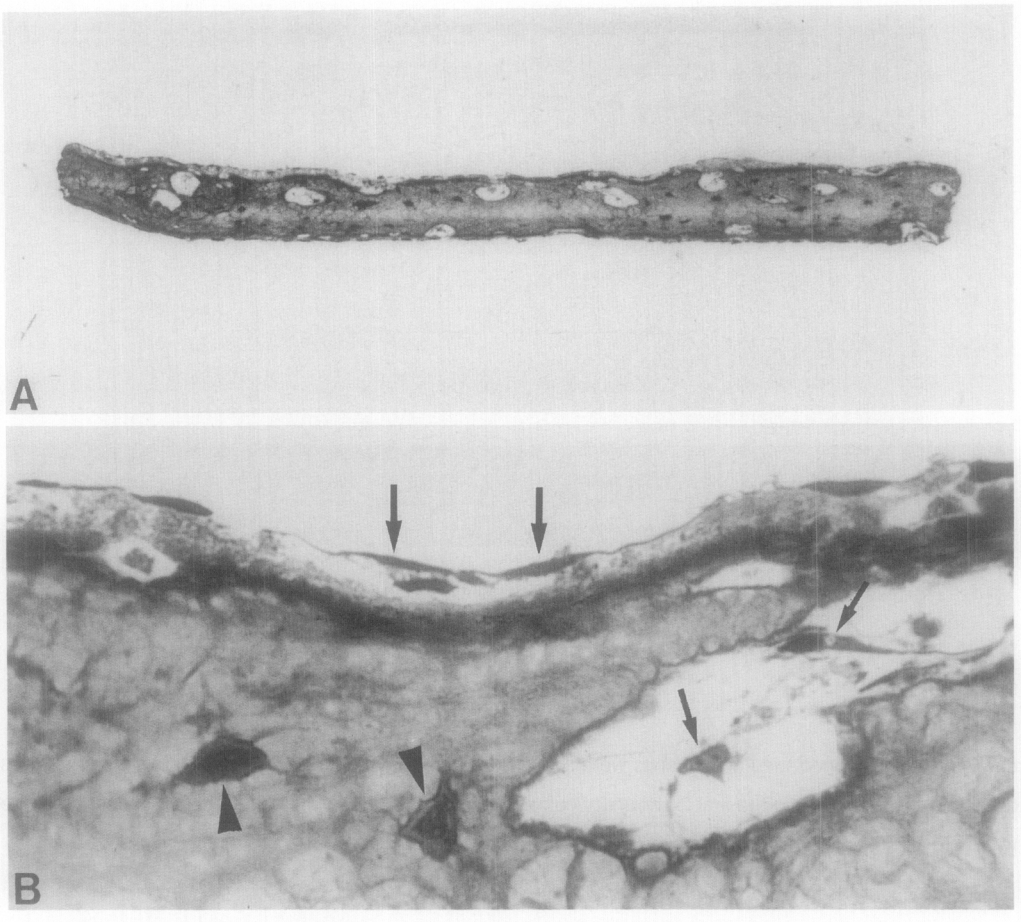

Fig. 2. Semithin section of 48-hour cultured periosteum-free calvarial fragment. (A) Low power picture showing the entire calvarial fragment surrounded by a new-formed cell layer, and containing osteocyte lacunae and vascular channels (170x). (B) A detail showing bone cells laying on the bone surface (large arrows), well preserved osteocytes (arrowheads) and cells located in the vascular channels (small arrows) (1900x).

Osteoclast bone resorption was significantly stimulated in co-culture, in the presence of calvarial fragments ($p<0.001$, Fig. 4A). Maximal effect was detectable at the end of the second 24 hours of co-culture when bone resorption resulted approximatively 183% compared to control. In order to evaluate whether the effect detected in periosteum-free calvarial fragments-osteoclasts co-cultures were due to osteoblasts, a set of experiments was performed co-culturing osteoclasts with isolated chick osteoblasts. Results (Fig. 4B) were similar to those described for calvarial fragments, indicating that even isolated bone forming cells activate osteoclast function.

Specificity was controlled co-culturing osteoclasts with periosteal cells (Fig. 4D), or skin fibroblasts (not shown). Results demonstrated no difference in bone resorption rate in co-cultures compared with osteoclasts alone, indicating that the effects shown for osteoblasts or for calvarial fragments were specific.

Finally, to control whether osteoblast-mediated osteoclast stimulation was due to soluble paracrine factors secreted into the medium, osteoclasts were incubated in the presence of 50% freshly harvested

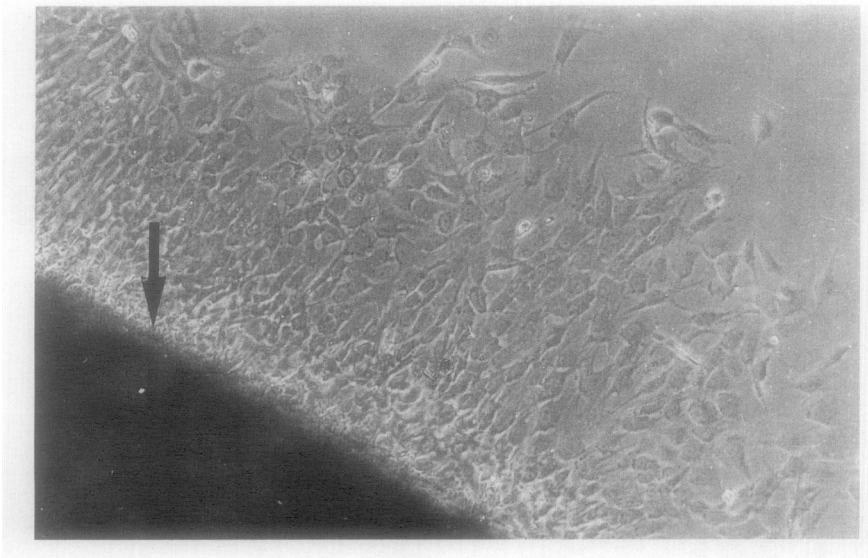

Fig. 3. Phase contrast micrograph showing cells migrating from collagenase digested chick calvarial fragment (arrow) at the 14th day of culture. (500x)

osteoblast conditioned medium. Results were similar to those above described (Fig. 4C) but the effect was detectable already during the first 24 hours of culture. This indicated that the simultaneous presence of the two cell types was not critical for stimulating osteoclast bone resorption.

Table 1. Osteoblast characterization

	CELLS MIGRATING FROM CALVARIA	PERIOSTEAL CELLS
Alkaline phosphatase activity		
nmol 4-MUMBF/min/mg protein	51.50±0.9	18.20±0.3
Osteocalcin production		
ng/mg protein		
Control	2.07±0.12	0.15±0.01
1,25 $(OH)_2D_3$ 10^{-8} M	5.40±0.31	ND
cAMP production		
nmol/mg protein		
Control	0.18±0.01	1.50±0.07
PTH 10^{-8} M	1.03±0.01	1.50±0.14
Type I collagen	+++	+++
Type III collagen	±	±

The table shows the characterization of cells migrating from collagenase-digested calvarial fragments according to osteoblastic phenotype parameters. Results indicated that these cells displayed high level of alkaline phosphatase activity, increased osteocalcin production in the presence of 1,25-dihydroxyvitamin D_3, increased cAMP production in response to PTH, and expressed high levels of type I collagen and very low levels of type III collagen. Periosteal cells failed to show differentiated osteoblastic phenotype.

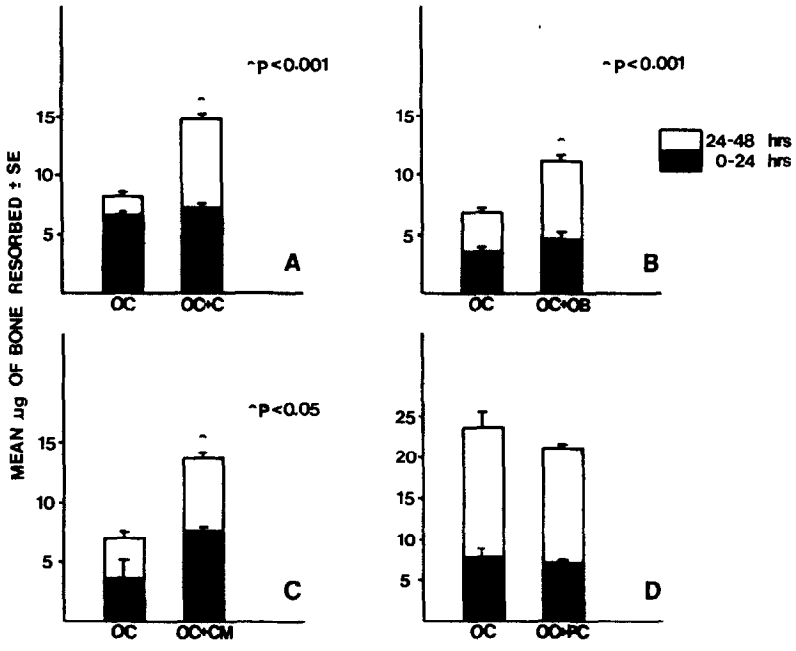

Fig. 4. Diagrams showing osteoclast bone resorption in co-culture in the presence of calvarial bone fragments (A), isolated osteoblasts (B), osteoblast conditioned medium (C) and periosteal cells (D). Bone resorption was measured as described in Methods and expressed as mean µg of bone resorbed ± SE. Calvarial fragments (OC+C), as well as isolated osteoblasts (OC+OB) and osteoblast conditioned medium (OC+CM) significantly enhanced osteoclast bone resorption compared to the respective controls represented by osteoclasts alone (OC), whereas no significant difference was found, in bone resorption rate in the presence of periosteal cells (OC+PC).

DISCUSSION

Results showed in the present report clearly demonstrated an important stimulatory effect of osteoblastic cells upon osteoclast resorptive activity. This effect was exerted either by bone cells included in their physiological environment, as suggested by the experiments performed in the presence of calvarial fragments, either by isolated osteoblasts. The effect was specific, since replacement of osteoblasts with less differentiated periosteal cells, or with skin fibroblasts did not affect osteoclast bone resorption.

Our results indicate that soluble paracrine factors might be responsible of the osteoblast-mediated effect. This is demonstrated i) by the peculiar co-culture conditions in which the two cell types were physically separated by the membrane, but could communicate via soluble factors capable of crossing membrane pores and ii) by the evidence of the stimulatory effect even when osteoclasts were incubated in the presence of osteoblast conditioned medium. In the latter circumstance the effect was detectable already during the first 24 hours of culture. This could indicate that the time-frame observed in co-culture before the effect became visible reflected the time necessary for the soluble factors to achieve the threshold concentration and for the assay to detect bone resorption.

Previous reports in the literature (11-16, 23), described osteoblast-mediated enhancement of osteoclast bone resorption in the presence of additional factors. Data indicating a constitutive role played by osteoblasts upon osteoclast activity were, however, lacking. Our experiments have been performed in serum-free media, in which, therefore, serum factors were absent. This demonstrate that osteoblast-osteoclast cooperation shown in this study must be ascribed to osteoblast constitutive function. It is known

that osteoblasts are capable of producing a number of molecules which act either as extracellular matrix, or as paracrine/ autocrine factors (24). Studies to demonstrate whether the constitutive osteoclast-stimulating factor is one of the known osteoblast products or a novel molecule are in progress.

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