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Functional and structural interactions between osteoblastic and preosteoclastic cells in vitro

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Abstract. Osteoblasts are involved in the bone resorption process by regulating osteoclast maturation and activity. In order to elucidate the mechanisms underlying osteoblast/preosteoclast cell interactions, we developed an in vitro model of co-cultured human clonal cell lines of osteoclast precursors (FLG 29.1) and osteoblastic cells (Saos-2), and evaluated the migratory, adhesive, cytochemical, morphological, and biochemical properties of the co-cultured cells. In Boyden chemotactic chambers, FLG 29.1 cells exhibited a marked migratory response toward the Saos-2 cells. Moreover, they preferentially adhered to the osteoblastic monolayer. Direct co-culture of the two cell types induced: (1) positive staining for tartrate-resistant acid phosphatase in FLG 29.1 cells; (2) a decrease of the alkaline phosphatase activity expressed by Saos-2 cells; (3) the appearance of typical ultrastructural features of mature osteoclasts in FLG 29.1 cells; (4) the release into the culture medium of granulocyte-macrophage colony stimulating factor. The addition of parathyroid hormone to the co-culture further potentiated the differentiation of the preosteoclasts, the cells tending to fuse into large multinucleated elements. These in vitro interactions between osteoblasts and osteoclast precursors offer a new model for studying the mechanisms that control osteoclastogenesis in bone tissue.

Key words: Osteoblasts – Preosteoclasts – Cell differentiation – Human

Introduction

Accumulating evidence from parabiosis experiments and bone-marrow transplantation in osteopetrosis indicates that osteoclasts are derived from hemopoietic precursors that are carried to the bone tissue by the circulation (Walker 1972; Coccia et al. 1980). Bone extracellular matrix is thought to contain several constituents capable of promoting migration of preosteoclasts to the bone surface (Malone et al. 1982; Wijngaert et al. 1988; Braidman et al. 1990). Moreover, implantation of devitalized bone particles at ectopic sites, i.e., subcutaneously (Bleiberg et al. 1992), or onto the chick chorionallantoic membrane (Webber et al. 1990; Osdoby et al. 1988) induces formation of multinucleated osteoclast-like cells in association with the bone surface. All these findings suggest the role of bone matrix and bone cells in recruiting osteoclast precursors.

Once having entered the bone tissue, the preosteoclasts differentiate toward the mature phenotype under the influence of the local microenvironment (Marks 1983). Cytokines, growth factors and as yet unidentified organic and inorganic matrix components contribute to the regulation of osteoclast differentiation (Dickson and Scheven 1989; Fuller and Chambers 1989). In addition, interactions of preosteoclasts with other bone cells, including osteoblasts and bone endothelial cells (Marshall et al 1986; Formigli et al. 1995), may also stimulate osteoclastogenesis. In vitro studies have shown the need of close contacts between osteoblasts and preosteoclasts for osteoclast maturation to occur (Takahashi et al. 1988a; Yamashita et al. 1990). In addition, multinucleated osteoclast-like cells appear only to form close to colonies of alkaline-phosphatase-positive bone-marrow cells, possibly representing osteoblasts (Takahashi et al. 1988b). Osteoblasts may therefore contribute to the differentiation of preosteoclasts into mature elements either through the secretion of soluble factors that induce marrow cell precursors to express the osteoclast phenotype (Greenfield et al. 1992; Dickson and Scheven 1989) and/or through direct cell-to-cell contacts. Several in vivo morphological studies showing that preosteoclast differentiation preferentially occurs in close proximity to osteoblasts are consistent with the latter hypothesis; moreover, specialized intercellular gap junctions form between the two cell types (Tran Van et al. 1982; Ejiri 1983; Irie and Ozawa 1990; Yamaga et al. 1992). Conversely, the more immature osteoclast precursors are mainly located around the blood vessels (Ejiri 1983; Luk et al. 1974).

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Despite the numerous observations on the potential role of osteoblasts in promoting osteoclast maturation, the mechanisms underlying the complex interaction between the two cell types remain to be fully understood. In order to test the functional and morphologic interactions between osteoblasts and preosteoclasts, we have developed a novel in vitro system of co-cultured clonal cell lines of human osteoclast precursor cells (FLG 29.1; Gattei et al. 1992) and osteoblastic cells (Saos-2; Rodan et al. 1987) and have evaluated the migratory responses, the adhesive properties, and the biochemical and morphologic interactions between the two cell types.

Materials and methods

Materials

Media and serum for cell culture were purchased from Gibco (Grand Island, N.Y.). Tissue culture plastic ware was obtained from Falcon (Oxnard, Calif.). Granulocyte-macrophage colony stimulating factor (GM-CSF) was detected using an enzyme-linked immunosorbed assay (ELISA) kit obtained from Medgenix Diagnostics (Fleurus, Belgium). Millicell-HA tissue culture plate well inserts were obtained from Millipore (Bedford, Mass., USA). Alkaline phosphatase activity was tested utilizing a commercially available kit from Sigma (St.Louis, Mo., USA); 12-O-tetradecanoylphorbol-13-acetate (TPA) was also obtained from Sigma. GM-CSF was provided by R & D Systems (Minneapolis, Minn., USA). Rat parathyroid hormone (rPTH) (1–84) was provided by Sigma.

Cell cultures

The FLG 29.1 clonal cell line was established from a 38-year-old female suffering from acute monoblastic leukemia. These cells are capable of differentiating toward the osteoclastic phenotype (Gattei et al. 1992; Formigli et al. 1995) in the presence of 10^{-7} M TPA (Cooper et al. 1982; Pegoraro et al. 1980) and in co-culture with bone endothelial cells. The cells were grown in RPMI 1640 culture medium, supplemented with 10% fetal calf serum (FCS) and gentamycin (100 μg/ml), in an atmosphere of 10% CO_2/90% air at 37°C.

The human osteosarcoma cell line Saos-2 was obtained from the American Type Culture Collection (ATCC, HTB85, Rockville, Md., USA) and grown in Coon's modified Ham's F12 medium supplemented with 10% FCS, 2 mM L-glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml). The cells were grown in a humidified atmosphere of 95% air/5% CO_2 at 37°C (Rodan et al. 1987).

The clonal bovine endothelial cell line (BBE) was cultured, as previously described, in Coon's modified Ham's medium containing 10% Nu-serum and 1% Ultroser-G (Streeten et al. 1989).

The human marrow stromal cells were isolated from healthy normal volunteers. A written declaration of consent was obtained from each volunteer. Fragments were incubated in minimum essential medium (MEM) containing 0.2% collagenase type IV at 37 °C in an atmosphere of 5% CO_2/95% air. After overnight enzymatic digestion, cells were mechanically dispersed with a 10-ml plastic pipette and centrifuged. The resulting pellet was plated in tissue culture flasks (10^5 cells/ml) and grown in MEM supplemented with 20% FCS, penicillin (100 IU/ml), and streptomycin (100 μg/ml).

The human articular cartilage cells were obtained from healthy normal volunteers. A written declaration of consent was obtained from each volunteer. Fragments were incubated in Coon's modified Ham's F12 medium containing 0.125% trypsin and 0.2% collagenase type IV at 37°C in an atmosphere of 5% CO_2/95% air. After overnight enzymatic digestion, cell aggregates were mechanically dispersed with a 10-ml plastic pipette and centrifuged. The resulting pellet was plated in tissue culture flasks (10 cells/ml) and grown in Coon's modified Ham's F12 medium supplemented with 10% FCS, L-glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml).

Fetal osteoblast-like cells were obtained from 8–12 week human fetal calvariae. Populations of fetal calvarial cells were prepared by collagenase digestion as described previously (Canalis 1983). Briefly, fetal calvariae were minced with scissors and digested with a 3 mg/ml solution of crude bacterial collagenase in magnetically stirred spinner flasks at 37 °C. Released cells were collected by centrifugation after 10 min and cultured in Coon's modified Ham's medium containing 10% FCS, penicillin (100 IU/ml), and streptomycin (100 μg/ml).

Chemotaxis

The chemotactic response of FLG 29.1 cells to various bone-derived cells was measured in blind-well Boyden chambers (Boyden 1962). In these experiments, a suspension of BBE cells or of Saos-2, human marrow stromal, and human articular cartilage cells was plated in each of the bottom wells in growth medium, and left to adhere for 24 h. After this time, the cell monolayers were washed twice in Coon's modified Ham's F12 medium without growth factors (steady state medium) and maintained in steady state medium containing 0.1% bovine serum albumin (BSA) for the duration of the experiment. The 48 lower wells were covered with a nucleopore membrane of 10 μm thickness, the 8-μm pores having been previously coated with a solution of gelatin (20 μg/ml).

FLG 29.1 cells were centrifuged in steady state medium containing 0.1% BSA. The cell suspension was then added to the top wells of the chambers and the chambers were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Steady state medium containing 0.1% BSA was used to determine background counts in six replicates. After 3 h at 37°C in air containing 5% CO_2, the membranes were removed from the chambers, fixed in methanol, stained with modified Wright's stain, and then counterstained and placed on microscopy slides with the surface containing migrated cells in contact with the slide. Non-migrated cells on the upper surface of the membranes were wiped off. The number of cells that had migrated to the lower surface of the filter was determined by counting the cell nuclei in 15 random fields at a magnification of 400×. Cells that remained viable during incubation were determined by their ability to exclude trypan blue. Results represent the mean ±SD of six experimental points.

Quantitative adhesion assay

BBE cells, Saos-2 cells, human articular cartilage cells, human marrow stromal cells, and human fetal bone cells were plated in a 24-multiwell plate at 2×10^5 cells/well in growth medium and left to adhere. After 24 h, the medium was removed; the cell monolayers were washed twice with steady state medium and were maintained in steady state medium containing 0.1% BSA for the duration of the experiment. FLG 29.1 cells were then added to each well at 10^5 cells/well and incubated for 24 h at 37°C in 5% CO_2. The non-adherent FLG 29.1 cells were then removed by gentle aspiration and each well was washed twice with steady state medium. The non-adherent cells were centrifuged at 1200×*g* for 10 min, resuspended in 1 ml steady state medium and counted. The percentage of cell adhesion was determined by the difference between the number of FLG 29.1 cells plated on the various cell monolayers at the beginning of the experiments and the number of FLG 29.1 cells found to be non-adherent to the endothelial cells at the end of the experiments. The experiments were carried out in triplicate and the results expressed as the mean ±SD of three different experiments.

Evaluation of multinucleated cell numbers

FLG 29.1 cells were co-cultured with Saos-2, BBE, bone-marrow and cartilage cells for 48 h. Cultures were then fixed in 80% ethanol and stained with hematoxylin and eosin. FLG 29.1 cells containing two or more nuclei were counted in 50 microscopy fields at 400×.

GM-CSF release

The amount of GM-CSF released by FLG 29.1 and Saos-2 cells alone or in co-culture was measured using a commercially available immunoenzymatic assay. FLG 29.1 (5×10^5) and Saos-2 (1×10^6) cells were cultured separately or in direct contact in 25 cm^2 flasks in the presence or absence of TPA. After a 24-h incubation, the medium was removed and replaced with serum-free medium for 48 h. The media were then collected, centrifuged at 1000×*g* for 15 min, and stored at –80°C until the assay. Experiments were carried out in four replicates and results were the mean ±SD of three different experiments.

Ultrastructural studies

The morphologic interactions between Saos-2 and FLG 29.1 cells were evaluated by transmission (TEM) and scanning (SEM) electron microscopy. For TEM analysis, 2×10^5 Saos-2 cells were cultured in growth medium on cellulose membranes in 24-well dishes for 24 h. FLG 29.1 cells (1.5×10^5 cells/well) were then added to the osteoblastic monolayers in a mixture of Coon's modified Ham's F12 and RPMI 1640 media (1:1) supplemented with 10% FCS, in the presence or absence of 10^{-8} M rPTH (1–84). After a 48-h incubation, the membranes with the adherent cells were fixed in 4% cold glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at room temperature and postfixed in 1% OsO_4 in 0.1 M phosphate buffer, pH 7.4, at 4 °C. The samples were then dehydrated in an acetone series, passed through propylene oxide, and embedded in Epon 812. Semi-thin sections (1–2 µm thick) were cut and stained with toluidine blue-sodium tetraborate. Ultrathin sections were also obtained from the same specimens, stained with uranyl acetate and alkaline bismuth subnitrate, and then examined under a Siemens Elmiskop electron microscope at 80 kV. For SEM analysis, the glass cover-slips bearing Saos-2 (2.5×10^5 cells) and FLG 29.1 cells (2.5×10^5 cells) alone and in co-culture were fixed in 2% glutaraldehyde in 0.05 M phosphate buffer, pH 7.2, with 0.02% $CaCl_2$, and postfixed in 1% OsO_4 in the same buffer containing 2% saccharose. After dehydration in alcohol and amyl-acetate, the specimens were critical-point dried and sputter-coated with 10% gold-palladium. Observations were performed under a Cambridge Stereoscan 100 SEM at an accelerating voltage of 15 kV and with a tilt angle varying from 20° to 40°.

Tartrate-resistant acid phosphatase activity

Tartrate-resistant acid posphatase (TRAcP) staining was performed on FLG 29.1 and Saos-2 cells co-cultured for 48 h. After fixation in 80% ethanol, the cultures were incubated with a solution containing naphthol AS-BI phosphate (Sigma) as a substrate for the reaction, sodium tartrate and 4% pararosaniline in 2 N HCl as a coupler, for 2 h. The cultures were then counterstained with hematoxylin, and TRAcP positive FLG 29.1 cells were counted. Experiments were carried out in triplicate.

Alkaline phosphatase activity

Saos-2 cells (1×10^6 cells) were cultured alone in growth medium and in co-culture with FLG 29.1 cells (5×10^5 cells) in a mixture of Coon's modified Ham's F12 and RPMI 1640 media (1:1) supplemented with 10% FCS in 6 well plates for 48 h, and then exposed to the steady state medium at 37°C for 24 h. The cells were washed twice with phosphate-buffered saline, scraped into 1 ml 1% Nonidet P-40, and sonicated for 5 min with a sonifier cell disruptor at 50 Watts, 20 kHz for 30 s. The sonicates were centrifuged for 15 min at 1000×*g*, and the supernatants were removed and stored at –80°C. Alkaline phosphatase activity was determined in thawed samples of the cell extracts. Enzyme activity in the lysate was corrected for cell number. Results were carried out in triplicate and expressed as the mean ±SD IU/10^6 cells.

Statistical analysis

All comparisons involved the use of Student's *t*-test of means. Data were expressed as mean ±SD.

Results

Saos-2 cells stimulated chemotaxis of undifferentiated FLG 29.1 cells in a cell-number-dependent manner, even

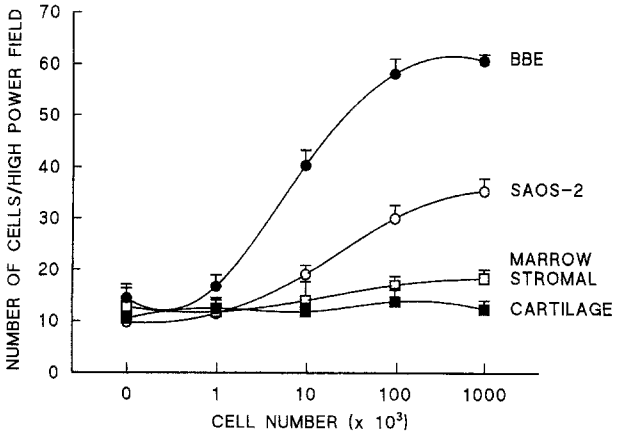

Fig. 1. Chemotactic responses of FLG 29.1 cells to different bone cell types (Saos-2, BBE, human marrow stromal, and articular cartilage cells). Cell numbers in a high-power field are given as a measurement of chemotaxis on the *ordinate* axis. Results represent the mean ±SD of six experimental points

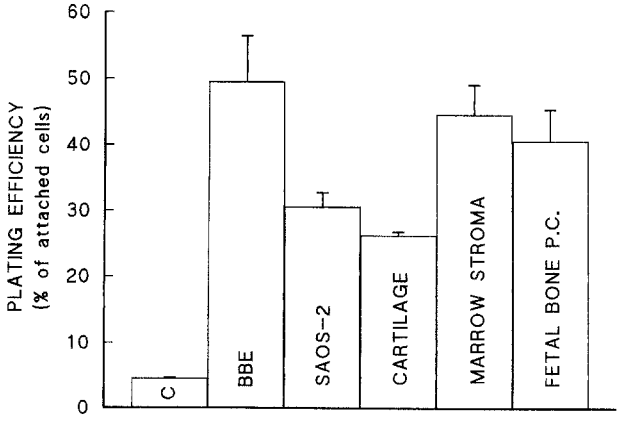

Fig. 2. Adhesion of FLG 29.1 to various cell types (BBE, Saos-2, human marrow stromal, articular cartilage, and fetal bone cells). The experiments were carried out in triplicate and the results were expressed as the mean ±SD of three different experiments

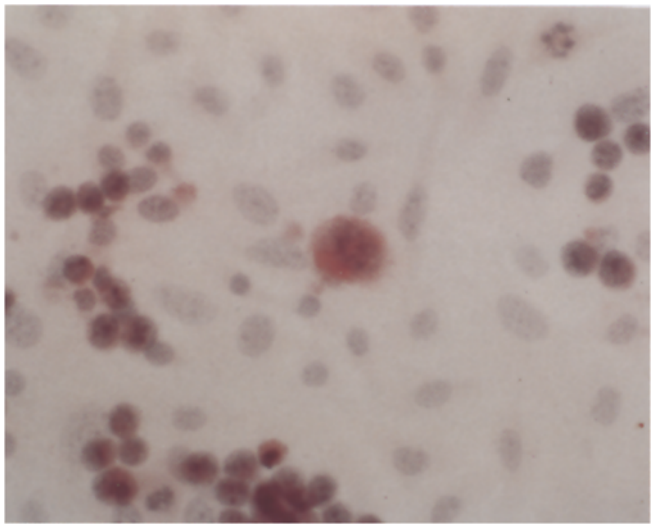

Fig. 3. FLG 29.1 co-cultured with Saos-2 cells. A strong TRAcP positive reaction in the FLG 29.1 cell is seen after two days of co-culture. ×140

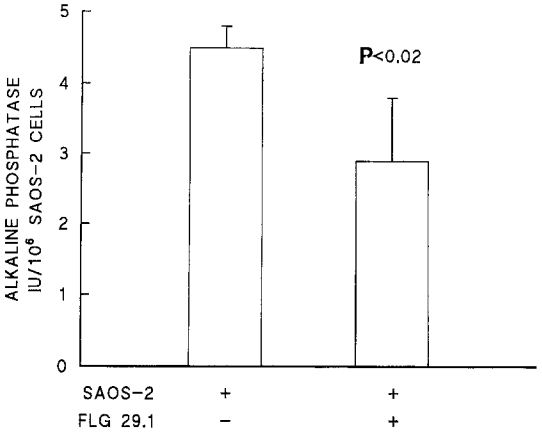

Fig. 4. Alkaline phosphatase activity in Saos-2 cells cultured alone and with FLG 29.1 cells. Experiments were carried out in triplicate and the results were expressed as the mean ±SD of three different experiments

if at a lower level than that promoted by BBE cells (Fig. 1). Conversely, human marrow stromal and articular cartilage cells in primary culture were unable to promote the migration of FLG 29.1 cells (Fig. 1).

Moreover, FLG 29.1 preferentially adhered to Saos-2 cell monolayers rather than to plastic surfaces (30±2.2% versus 4.0±0.1% attached cells; $P<0.001$) (Fig. 2). The cells also revealed a significant plating efficiency on other bone cell types, such as BBE cells ($P<0.001$), primary cultures of human articular cartilage ($P<0.002$), bone-marrow stromal cells ($P<0.001$), and fetal bone-derived cells ($P<0.005$).

Direct co-culture of the two cell types for 48 h induced a strong TRAcP-positive stain in FLG 29.1 cells (Fig. 3) and significantly reduced the intracellular content of alkaline phosphatase compared with that of Saos-2 cells cultured alone (2.9±0.8 vs 4.6±0.3 IU/10^6, $P<0.02$) (Fig. 4).

In the ultrastructural SEM analysis, FLG 29.1 cells, as single cells or in small clusters, appeared closely adherent to the underlying osteoblastic cells. Saos-2 cells exhibited a flattened and spread morphology with short microvillous processes at the apical surfaces (Fig. 5). FLG 29.1 cells possessed long thin cytoplasmic processes that seemed to improve their adhesion to the underlying osteoblastic cells (Fig. 5). The TEM investigation showed that FLG 29.1 in co-culture with Saos-2 cells displayed early ultrastructural signs of cellular differentiation toward a more mature phenotype. Indeed, when FLG 29.1 cells were cultured alone, they had typical morphologic features of undifferentiated monocytic cells, namely they were round, had a smooth cell surface, and their cytoplasm contained abundant free ribosomes, scarce membranous organelles, a large irregular nucleus with dispersed chromatin, and a prominent nucleolus (Fig. 6A). Conversely, FLG 29.1 cells in co-culture with Saos-2 cells exhibited a better differentiated cytoplasm with several profiles of rough endoplasmic reticulum (RER) and a well developed Golgi complex with primary lysosomes nearby (Fig. 6B,C). Sites of adhesions in the form of dark

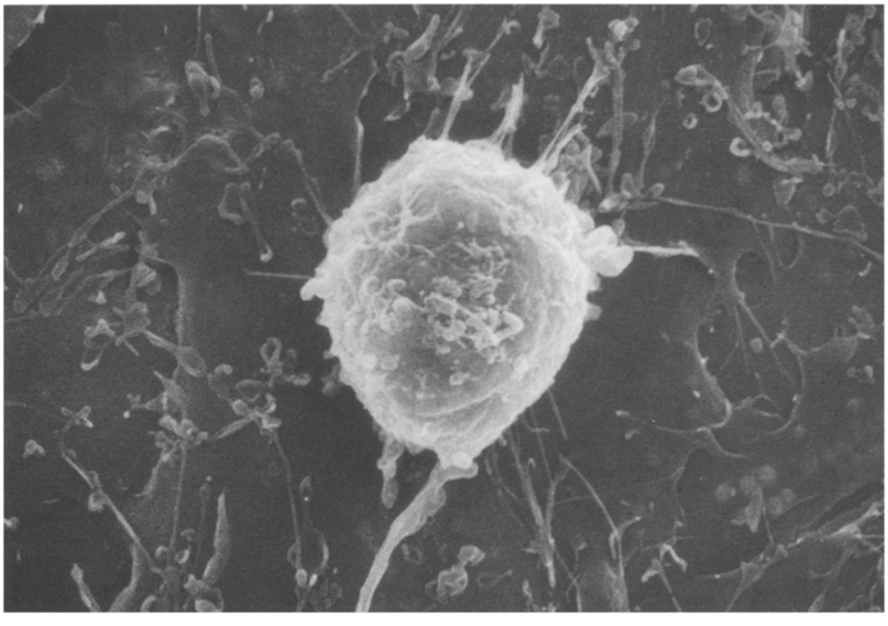

Fig. 5. Scanning electron microscopy of FLG 29.1 cells co-cultured with Saos-2 cells. In areas of FLG 29.1/Saos-2 cell interaction, elongated cytoplasmic processes arising from an FLG 29.1 cell seem to potentiate adhesion to the underlying Saos-2 cells. ×3000

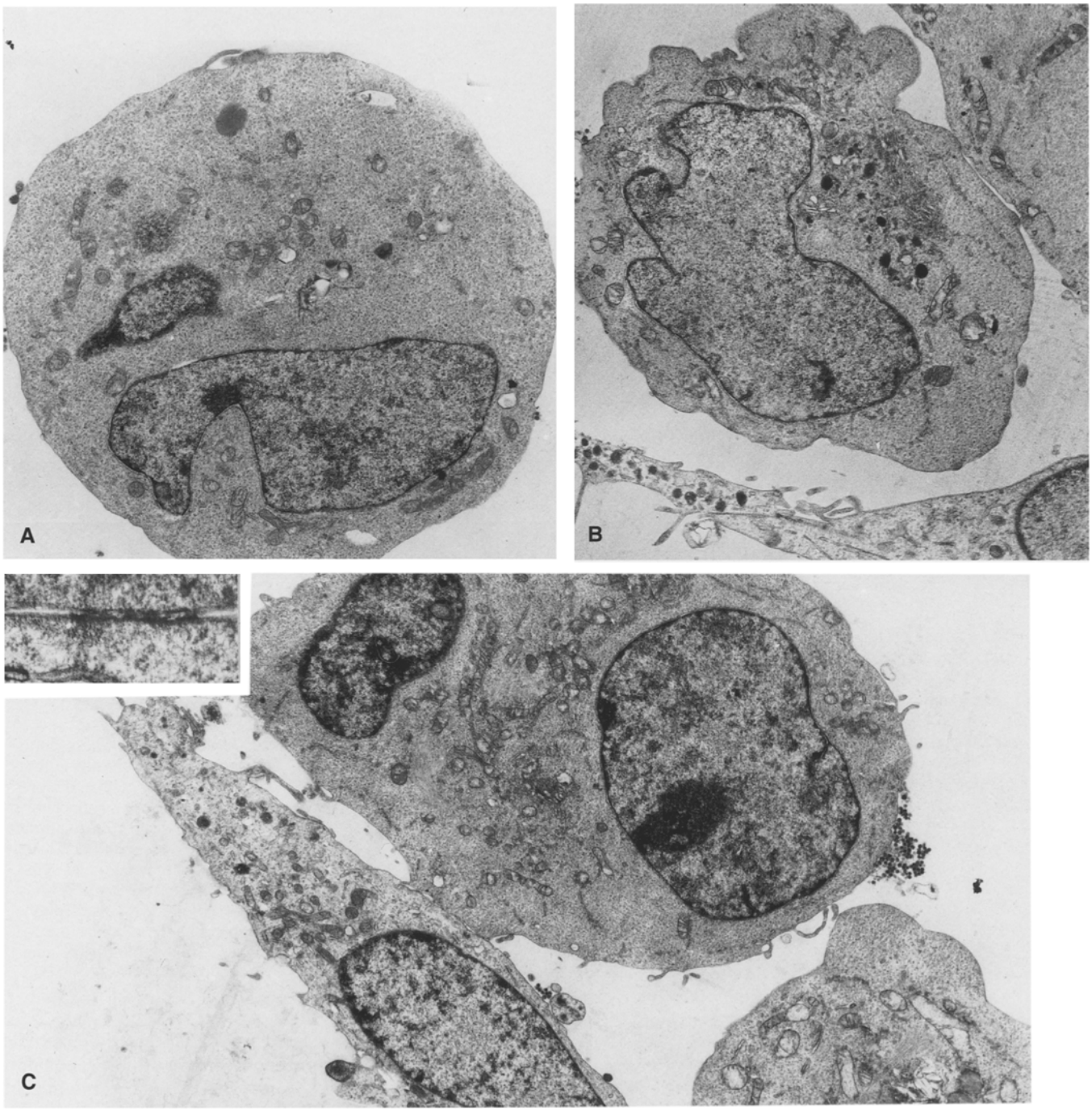

Fig. 6A–C. Transmission electron microscopy of FLG 29.1 cells cultured alone or with Saos-2 cells. **A** FLG 29.1 cell cultured alone. The cytoplasm shows few organelles other than numerous free ribosomes. ×7500. **B** FLG 29.1 cells co-cultured with Saos-2 cells. The cytoplasm of the FLG 29.1 cells shows numerous organelles including several RER profiles, a Golgi complex and primary lysosomes. ×6500. **C** FLG 29.1 cells co-cultured with Saos-2 cells. A large FLG 29.1 cell with two peripherally displaced nuclei has established sites of adhesion to the underlying osteoblastic cell. ×5500. In the *insert*, a detail of the adhesion area between the two cell types can be seen with electron-dense "feet" interposed between the plasma membranes. ×25000

"feet", containing an electron-dense amorphous material, could be encountered at sites of close apposition of the two cell types (Fig. 6C, insert). The addition of 10^{-8} M rPTH (1–84) to the co-culture resulted in a tendency for the preosteoclastic cells to fuse together into large multinucleated cells. Adjacent FLG 29.1 cells displayed: (1) areas of close apposition of the plasma membranes; (2) intercellular junctional complex formation, that appeared to be broken down in certain areas (Fig. 7A); and (3) the disappearance of some apposed plasma membranes with complete cytoplasmic continuity (Fig. 7B). Cup-like structures were seen in the cell membrane bordering the regions of fusion (Fig. 7B). By SEM, groups of fused FLG 29.1 cells could be easily encountered in the co-culture after the addition of rPTH. These clusters revealed peripheral cytoplasmic laminar expansions that seemed

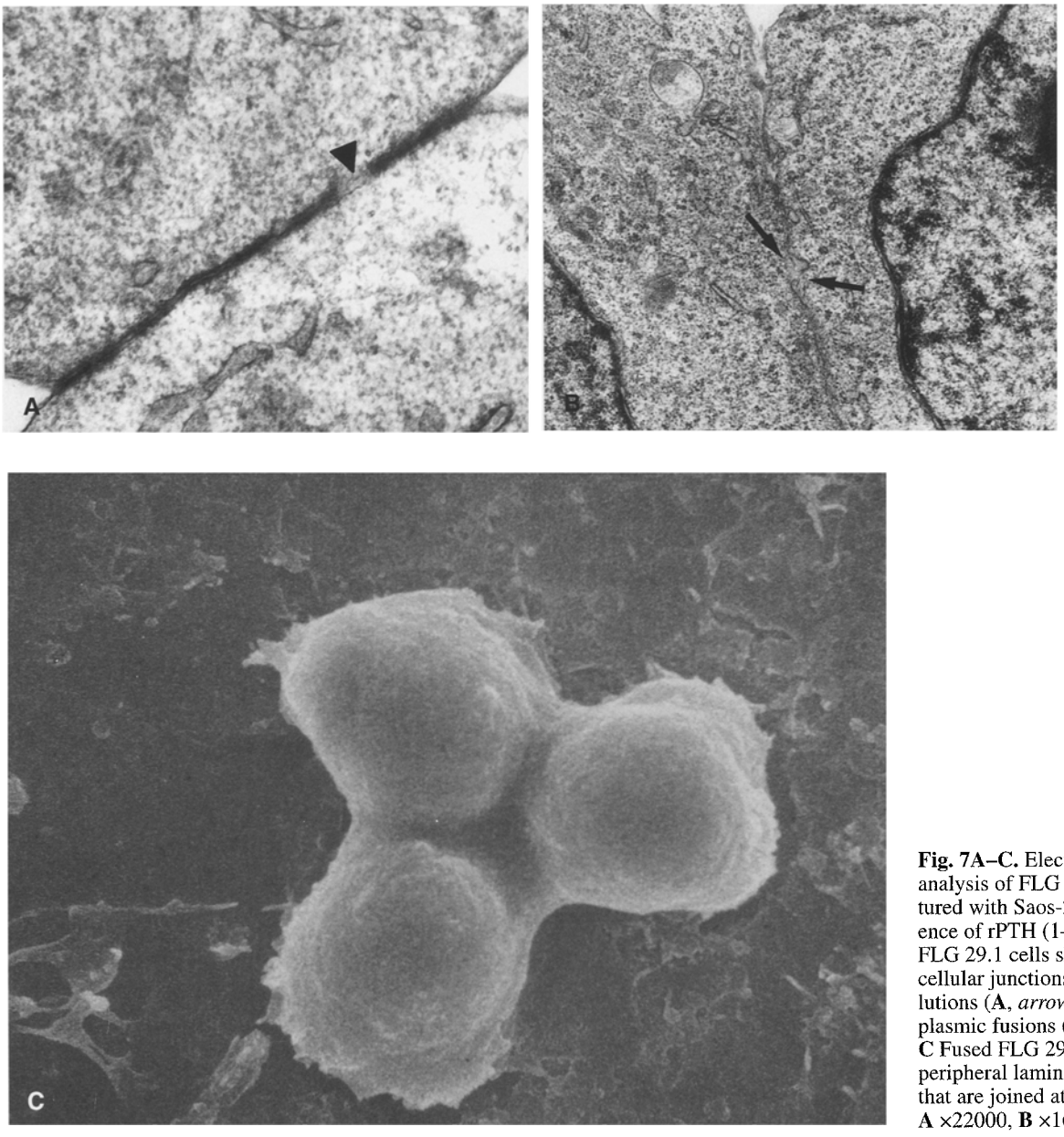

Fig. 7A–C. Electron-microscopic analysis of FLG 29.1 cells co-cultured with Saos-2 cells in the presence of rPTH (1–84). Adjacent FLG 29.1 cells show areas of intercellular junctions with focal dissolutions (**A**, *arrowhead*) and cytoplasmic fusions (**B**, *arrows*). **C** Fused FLG 29.1 cells display peripheral laminar expansions that are joined at the fusion area. **A** ×22000, **B** ×16000, **C** ×3800

to increase the contact area with the underlying Saos-2 cells and with adjacent FLG 29.1 cells (Fig. 7C).

The number of multinucleated FLG 29.1 cells increased significantly when co-cultured with Saos-2 cells (0.59±0.07 vs 0.13±0.02, $P<0.004$) and with BBE cells (0.39±0.02 vs 0.13±0.02 $P<0.0002$) (Fig. 8). No statistical differences in the number of multinucleated FLG 29.1 cells were found when the cells were co-cultured with bone-marrow or cartilage cells (Fig. 8). Moreover, cells with better developed organelles, including numerous pleomorphic mitochondria and clear vesicles, were sometimes encountered in the co-culture after the addition of rPTH (Fig. 9). In these cells, peripheral areas devoid of cell organelles and filled with fine filaments could be observed (Fig. 9).

In order to analyze the molecular mechanisms underlying the osteoblast-preosteoclast cell interactions the release of GM-CSF by the two cell types was measured. Neither FLG 29.1 nor Saos-2 cells released measurable amounts of GM-CSF into the culture medium. The addition of TPA induced the release of small amounts of GM-CSF by the two cell types (1.12±0.13 ng/ml for FLG 29.1, $P<0.0005$, and 0.21±0.01 ng/ml for Saos-2 cells, $P<0.005$) (Fig. 10). However, when the two cell types were co-cultured in direct contact, the amount of GM-CSF secreted increased significantly (1.48±0.34 ng/ml, $P<0.001$ vs control media) (Fig. 10); this effect was potentiated by the addition of 10^{-7} M TPA (2.64±0.4 ng/ml, $P<0.05$ vs untreated co-culture) (Fig. 10).

Discussion

The mechanisms that regulate osteoclast development and function during bone growth and remodeling have

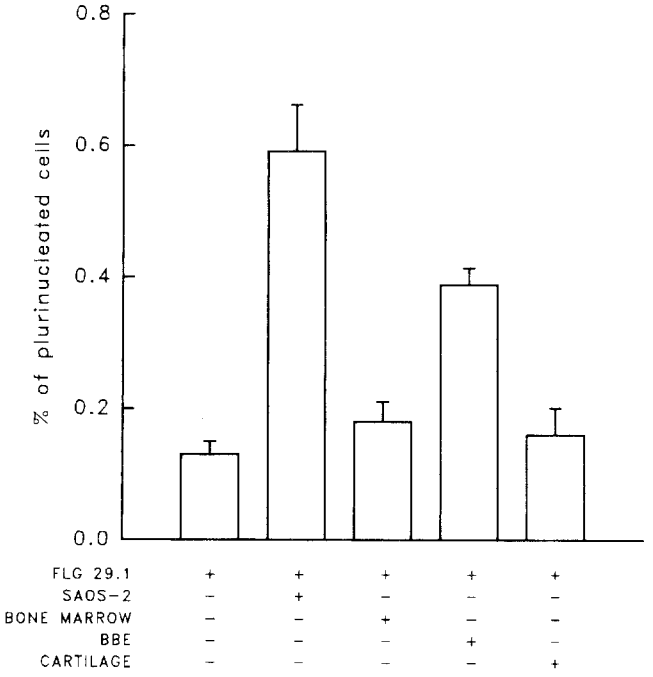

Fig. 8. Percentage of multinucleated FLG 29.1 cells in co-culture with various bone cell types (Saos-2, BBE, human bone marrow, and cartilage cells). The experiments were carried out in triplicate and the results were expressed as the mean ±SD of three different experiments

not been fully characterized. Nevertheless, numerous studies indicate that osteoblasts play an important role in the process of bone resorption via: (1) the digestion of the organic material covering the bone surface and subsequent exposure of the mineralized matrix to the osteoclast (Chambers and Fuller 1985); (2) the activation of pre-existing osteoclasts in the presence of PTH or vitamin D3 (Thomson et al. 1986; McSheehy and Chambers 1986a, b; Teti et al.1991; Perry and Gurbani 1992); and (3) the regulation of osteoclast differentiation from immature bone-marrow progenitor cells (Burger et al. 1984; Marshall et al. 1986; Takahashi et al. 1988a; Dickson and Scheven 1989). Recently, it has been suggested that osteoblasts, besides secreting soluble factors able to activate quiescent osteoclasts, retain them on their cell surface or adjacent extracellular matrix, thus contributing to a reservoir of these factors that can then be made available during the bone remodeling process (Fuller et al. 1991). Despite the abundant evidence indicating functional interactions between osteoblasts and osteoclasts, the biochemical mechanisms and the ultrastructural modifications underlying such complex relationships remain to be elucidated.

The present findings obtained by direct co-culture of clonal cell lines of an osteoblastic and preosteoclastic nature, may contribute to clarifying some of the mechanisms by which osteoblasts influence preosteoclast recruitment and maturation. Indeed, osteoblastic cells appear to stimulate the migration of the preosteoclastic FLG 29.1 cells. This finding indicates that osteoblasts play a role in the recruitment of osteoclast precursors into the bone tissue, a role ascribed until now to bone matrix constituents and recently to bone endothelial cell (Formigli et al. 1995). FLG 29.1 cells also adhere to the underlying osteoblastic cell monolayers with the formation of specialized intercellular junctions. Under these conditions, preosteoclasts show better developed organelles than those of FLG 29.1 cells cultured alone, in particular with respect to their well-extended Golgi apparatus and the primary lysosomes and clear vesicles nearby. All these ultrastructural findings have been previously reported for osteoclast precursors differentiating in vivo toward

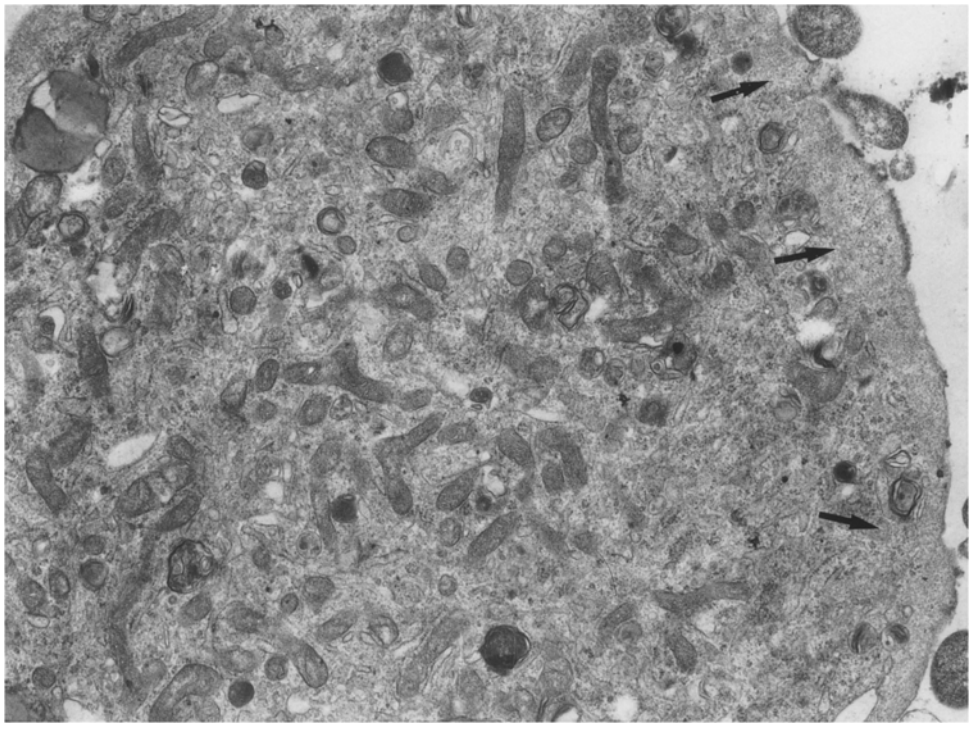

Fig. 9. FLG 29.1 cells co-cultured with Saos-2 cells in the presence of 10^{-8} M rPTH (1–84). The cytoplasm of a FLG 29.1 cell shows abundant organelles including branched mitochondria and clear vesicles. The outer cytoplasmic rim is devoid of organelles and is filled with thin filaments (*arrows*). ×15000

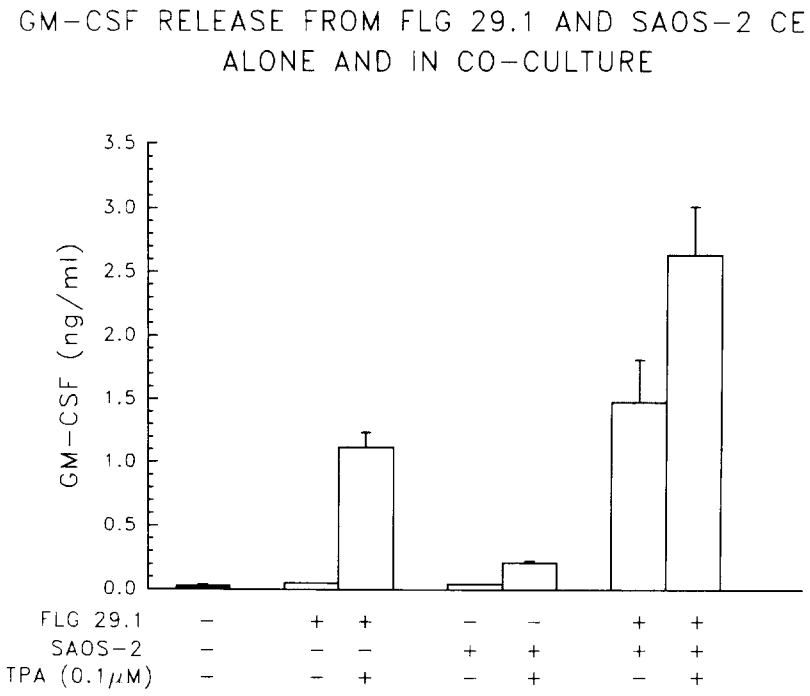

Fig. 10. GM-CSF release from FLG 29.1 and Saos-2 cells alone and in co-culture in the presence or absence of TPA. Experiments were carried out in triplicate and the results were expressed as the mean ±SD of three different experiments

a more mature phenotype (Ejiri 1983; Luk et al. 1974). Interactions with osteoblasts are also necessary for preosteoclasts to develop positive staining for TRAcP, an enzyme expressed in vivo by mature osteoclasts (Hattersley and Chambers 1989). It is conceivable that a direct contact between osteoblasts and preosteoclasts may be required for osteoclast maturation. Interestingly, the acquisition of these distinctive parameters by the preosteoclastic cells is correlated with a decrease of the alkaline phosphatase content in the osteoblastic cells, the presence of which enzyme in osteoblasts is considered to be a functional parameter associated with new bone formation (Gehron Robey 1989). This finding suggests the existence of a role for osteoclast precursors in osteoblast biosynthetic activity and/or mineralization, and confirms the results previously obtained showing that conditioned media from osteoclasts are able to inhibit the synthesis of collagen and the alkaline phosphatase activity of osteoblasts (Galvin and Osdoby 1991; Formigli et al. 1991).

The addition of PTH to the co-culture further potentiates the differentiation of the preosteoclastic cells, namely by increasing their tendency to fuse together into large multinucleated elements. The process of fusion of the preosteoclastic cells may be considered a further step in the differentiation to the osteoclastic phenotype, since these cells have been demonstrated to form by the fusion of mononuclear precursors (Baron et al. 1986; Kurihara et al. 1990). Moreover, in the presence of PTH, the preosteoclastic cells exhibit some of the typical ultrastructural features of mature osteoclasts, including a highly vacuolated cytoplasm that is rich in pleomorphic mitochondria, but that is provided with a peripheral area poor in organelles. The PTH effect on osteoclast differentiation is consistent with the well-known role that this hormone plays in osteoclast formation (Ibbotson et al. 1984; McDonald et al. 1987; Akatsu et al. 1989; Takahashi et al. 1988a, b; Hattersley and Chambers 1989). It is generally believed that PTH stimulates osteoclast formation indirectly via a primary action on osteoblasts (McSheehy and Chambers 1986 a, b), since these cells, unlike osteoclasts, express PTH receptors. This could also be the case in the present model, where PTH is not able to modify either the morphology or the function of FLG 29.1 cells cultured alone (L. Formigli, personal communication).

We have also characterized the regulation of GM-CSF release by the two cell types in co-culture. Previous studies have demonstrated that a large amount of this cytokine is secreted by cultured osteoblastic cells upon stimulation with PTH or tumor necrosis factor α (Horowitz et al. 1989; Felix et al. 1991). The production of this growth factor, which is known to stimulate immature hematopoietic cells to proliferate and differentiate into mature granulocytes and macrophages (Metcalf 1986; Clark and Kamen 1987), may have several implications for bone tissue (Felix et al. 1991). Indeed, GM-CSF might contribute to the regulation of the hematopoiesis process taking place in the bone-marrow compartment. Alternatively, GM-CSF may be involved in the defense mechanisms occurring during severe inflammatory bone disease and/or in new bone formation by osteoblasts (Dedhar et al. 1988). The present findings of an increased GM-CSF production in a co-culture system, a condition essential for osteoclast maturation, suggest a further role for the growth factor in the biology of osteoclastogenesis. In particular, GM-CSF may represent a local factor involved in the bone resorption process by stimulating osteoclast generation from immature precursors. Consistent with these findings are recent studies indicating an effect of this growth factor on osteoclast cell formation in culture (Mochizuki et al. 1992; Hiura et al. 1991; Takahashi et al. 1991).

In conclusion, our results indicate that osteoblasts play an important role in the process of bone resorption being involved in a complex series of events including chemotaxis, adhesion, differentiation, and fusion of osteoclast precursors. Although the local bone microenvironment is more complicated than our simple co-culture system, the present model may represent a novel approach for dissecting the various cell-to-cell interactions that constitute the basis of the bone remodeling process.

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References

Akatsu T, Takahashi N, Udagawa N, Sato K, Nagata N, Moseley JM, Martin TJ, Suda T (1989) Parathyroid hormone (PTH)-related protein is a potent stimulator of osteoclast-like multinucleated cell formation to the same extend as PTH in mouse marrow cultures. Endocrinology 125:20–27

Baron R, Neff L, Tran Van P, Nefussi JR, Vignery A (1986) Kinetic and cytochemical identification of osteoclast precursors and their differentiation into multinucleated osteoclasts. Am J Pathol 122:363–378

Bleiberg I, Glowacki J,Anklesaria P, Greenberger JS (1992) Origin of stromal cells associated with osteoclast recruitment in s.c. implants of bone particles in chimeric mice. Exp Hematol 20:957–961

Boyden S (1962) The chemotactic effect of a mixture of antibody and antigen on polymorphonuclear leukocytes. J Exp Med 115:453–466

Braidman IP, Rothwell C, Webber DM, Crowe P, Anderson DC (1990) Location of osteoclast precursors in fetal rat calvaria cultured on collagen gel. J Bone Miner Res 5:287–298

Burger EH, Van Der Meer JWM, Nijweide PJ (1984) Osteoclast formation from mononuclear phagocytes: role of bone-forming cells. J Cell Biol 99:1901–1906

Canalis E (1983) Effect of glucocorticoids on type I collagen synthesis, alkaline phosphatase activity, and deoxyribonucleic acid content in cultured rat calvariae. Endocrinology 112:931–939

Chambers TJ, Fuller K (1985) Bone cells predispose bone surfaces to resorption by exposure of mineral to osteoclastic contact. J Cell Sci 76:155–165

Clark SC, Kamen R (1987) The human hematopoietic colony-stimulating factors. Science 236:1229–1237

Coccia PF, Krivit W, Cervenka J, Clawson C, Kersey JK, Kim TH Nesbit ME, Ramsay NKC, Warkentin PI, Teitelbaum SL, Kahn AJ, Brown DM (1980) Successful bone-marrow transplantation for infantile malignant osteopetrosis. N Engl J Med 302:701–708

Cooper RA, Braunwald AD, Kuo AL (1982) Phorbol ester induction of leukemic cell differentiation is a membrane-mediated process. Proc Natl Acad Sci USA 79:2865–2869

Dedhar S, Gaboury L, Galloway P, Eaves C (1988) Human granulocyte-macrophage colony stimulating factor is a growth factor active on a variety of cell types of nonhemopoietic origin. Proc Natl Acad Sci USA 85:9253–9257

Dickson IR, Scheven BAA (1989) Regulation of new osteoclast formation by a bone cell-derived macromolecular factor. Biochem Biophys Res Commun 159:1383–1390

Ejiri S (1983) The preosteoclast and its cytodifferentiation into the osteoclast: ultrastructural and histochemical studies of rat fetal parietal bone. Arch Histol Jpn 46:533–556

Felix, Cecchini MG, Hofstetter W, Guenther HL, Fleisch H (1991) Production of granulocyte-macrophage (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) by rat clonal osteoblastic cell population CRP10/30-myc1 stimulated by tumor necrosis factor. Endocrinology 126:661–667

Formigli L, Benvenuti S, Calabresi E, Paoletti C, Masi L, Bernabei PA, Brandi ML (1991) Differentiation of osteoclast precursors induced by osteoblastic cells. J Bone Miner Res 6:S94

Formigli L, Zecchi Orlandini S, Benvenuti S, Masi L, Pinto A, Gattei V, Bernabei PA, Gehron Robey P, Collin-Osdoby, Brandi ML (1995) "In vitro" structural and functional relationships between preosteoclastic and bone endothelial cells: a juxtacrine model for migration and adhesion of osteoclast precursors. J Cell Physiol (in press)

Fuller K Chambers TJ (1989) Bone matrix stimulates osteoclastic differentiation in cultures of rabbit bone-marrow cells. J Bone Miner Res 4:179–183

Fuller K, Gallagher AC, Chambers TJ (1991) Osteoclast resorption-stimulating activity is associated with the osteoblast cell surface and/or the extracellular matrix. Biochem Biophys Res Commun 181:67–73

Galvin RJS, Osdoby P (1991) Chick "osteoclast like" cell conditioned media inhibits osteoblast collagen synthesis and alkaline phosphatase activity. J Bone Miner Res 6[Suppl 1]:S143

Gattei V,Bernabei PA, Pinto A, Bezzini R, Ringressi A, Formigli L, Tanini A, Attadia V, Brandi ML (1992) Phorbol ester induced osteoclast-like differentiation of a novel human leukemic cell line (FLG 29.1). J Cell Biol 116:437–447

Gehron Robey P (1989) The biochemistry of bone. In Endocrinol Metab Clin North Am 18:859–897

Greenfield EM, Alvarez JI, McLaurine EA, Ousler MJ,Blair HC,Osdoby P,Teitelbaum SL, Ross FP (1992) Avian osteoblast conditioned media stimulate bone resorption by targeting multinucleated osteoclast precursors. Calcif Tissue Int 51:317–323

Hattersley G, Chambers TJ (1989) Generation of osteoclastic function in mouse bone-marrow cultures: multinuclearity and tartrate-resistant acid phosphatase are unreliable markers for osteoclastic differentiation. Endocrinology 124:1689–1696

Hiura K, Sumitani K, Kawata T, Higashino K, Okawa M,Sato T,Hakeda Y, Kumegawa M (1991) Mouse osteoblastic cells (MC3T3-E1) at different stages of differentiation have opposite effects on osteoclastic cells formation. Endocrinology 128:1630–1637

Horowitz MC, Coleman DL, Ryaby JT, Einhorn TA (1989) Osteotropic agents induce the differential secretion of granulocyte-macrophage colony stimulating factor by the osteoblast cell line MC3T3-E1. J Bone Miner Res 4:911–921

Ibbotson KJ, Roodman GD, McManus LM, Mundy GR (1984) Identification and characterization of osteoclast-like cells and their progenitors in cultures of feline marrow mononuclear cells. J Cell Biol 99:471–480

Irie K, Ozawa H (1990) Relatioship between tooth eruption, occlusion and alveolar bone resorption: histochemical and cytological studies of bone remodelling on rat incisor alveolar bone facing the enamel after root resection. Arch Histol Cytol 53:511–522

Kurihara N, Chenu C, Miller M, Civin C, Rodan GD (1990) Identification of committed mononuclear precursors for osteoclast-like cells formed in long term human marrow cultures. Endocrinology 126:2733–2741

Luk SC, Nopajaroonsri C, Simon GT (1974). The ultrastructure of endosteum: a topographic study in young adult rabbits. J Ultrastruct Res 46:165–183

Malone JD, Teitelbaum SL, Griffin GL, Senior RM, Kahn AJ (1982) Recruitment of osteoclast precursors by purified bone matrix constituents. J Cell Biol 92:227–230

Marks SC (1983) The origin of osteoclasts. J Oral Pathol 12: 226–256

Marshall MJ, Nisbet NW, Green PM (1986) Evidence for osteoclast production in mixed bone cell cultures. Calcif Tissue Int 38:268–274

McDonald BR, Takahashi N, McManus LM, Holahan J, Mundy GR, Roodman GD (1987) Formation of multinucleated cells

that respond to osteotropic hormones in long term human bone marrow cultures. Endocrinology 120:2326–2333

McSheehy PMJ, Chambers TJ (1986a) Osteoblastic cells mediate osteoclastic responsiveness to parathyroid hormone. Endocrinology 118:824–828

McSheehy PMJ, Chambers TJ (1986b) Osteoblast-like cells in the presence of parathyroid hormone release soluble factor that stimulates osteoclastic bone resorption. Endocrinology 119: 1654–1659

Metcalf D (1986) The molecular biology and function of the granulocyte-macrophage colony stimulating factors. Blood 67: 257–267

Mochizuki H, Hakeda Y, Wakatsuki N, Usui N, Akashi S, Sato T, Tanaka K, Kumegawa M (1992) Insulin-like growth factor I supports formation and activation of osteoclasts. Endocrinology 131:1075–1080

Osdoby P, Oursler MJ, Salino-Hugg T, Krukowski M (1988) Osteoclast development: the cell surface and the bone enviroment. Ciba Found Symp 136:108–124

Pegoraro L, Abrham JL, Cooper RA, Levis A, Lange B, Meo P, Rovera G (1980) Differentiation of human leukemias in response to 12-O-tetradecanoylphorbol-13-acetate in vitro. Blood 55:859–862

Perry HM, Gurbani S (1992) Development of monoclonal antibodies to parathyroid hormone-induced resorptive factors from osteoblast-like cells. Calcif Tissue Int 50:237–244

Rodan SB, Imai Y, Thiede MA, Wesolowski G, Thompson D, Bar-Shavit Z, Shull S, Mann K, Rodan GA (1987) Characterization of a human osteosarcoma cell line (Saos-2) with osteoblastic properties. Cancer Res 47:4961–4966

Streeten E, Ornberg R, Curcio F, Sakaguchi K, Marx S, Auerbach GB, Brandi ML (1989) Cloned endothelial cells from fetal bovine bone. Proc Natl Acad Sci USA 86:916–920

Takahashi N, Akatsu T, Udagawa N, Sasaki T, Yamaguchi A, Moseley JM, Martin JT, Suda T (1988a) Osteoblastic cells are involved in osteoclast formation. Endocrinology 123:2600–2602

Takahashi N, Yamana H, Yoshiki S, Roodman GD, Mundy GR, Jones SJ, Boyde A, Suda T (1988b) Osteoclast-like cell formation and its regulation by osteotropic hormones in mouse bone marrow cultures. Endocrinology 122:1373–1382

Takahashi N, Udagawa N, Akatsu T, Tanaka H, Shionome M, Suda T (1991) Role of colony-stimulating factors in osteoclast development. J Bone Miner Res 6:977–985

Teti A, Grano M, Colucci S, Cantatore FP, Loperfido MC, Zambonin Zallone A (1991) Osteoblast-osteoclast relationships in bone resorption: osteoblasts enhance osteoclast activity in a serum-free co-culture system. Biochem Biophys Res Commun 179:634–640

Thomson BM, Saklatvala J, Chambers TJ (1986). Osteoblasts mediate interleukin I stimulation of bone resorption by rat osteoclasts. J Exp Med 164:104–112

Tran Van P, Vignery A, Baron R (1982) An electron-microscopic study of bone-remodeling sequence in the rat. Cell Tissue Res 225:283–292

Van De Wijngaert FP, Schipper CA, Tas MC, Burger EH (1988) Role of mineralizing cartilage in osteoclast and osteoblast recruitment. Bone 9:81–88

Walker DG (1972) Congenital osteopetrosis in mice cured by parabiotic union with normal siblings. Endocrinology 91:916–920

Webber DM, Menton D, Osdoby P (1990) An in vivo model system for the study of avian osteoclast recruitment and activity. Bone Miner 11:127–140

Yamaga M, Iwaku M, Ozawa H (1992) Alveolar bone remodelling in the early stage of experimental apical periodontitis in the rat mandible. Arch Histol Cytol 55:137–150

Yamashita T, Asano K, Takahashi N,Akatsu T,Udagawa N, Sasaki T, Martin TJ, Suda T (1990). Cloning of an osteoblastic cell line involved in the formation of osteoclast-like cells. J Cell Physiol 145:587–595