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ARTICLES

Myeloblastic Cell Line Expresses Osteoclastic Properties Following Coculture With Marrow Stromal Adipocytes

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Abstract Osteoclasts are derived from hemopoietic precursors in the marrow. Their differentiation pathway is still undefined, but an important role was observed for the marrow microenvironment in the regulation of osteoclastogenesis. Various marrow stromal cell subtypes were used to study their possible role in the formation of osteoclasts from myeloblast (M1) cells. Interactions between M1 cells and the 14F1.1 endothelial-adipocyte stromal cell line were demonstrated in a coculture model. M1 cells attached to the adherent layer of 14F1.1 cells and formed distinct foci reminiscente of "cobblestone areas." Following these interactions, M1 cells developed specific enzymatic activities and became multinucleated. Both mononuclear and multinuclear M1 cells became positive to tartrate-resistant acid phosphatase (TRaP) and ATPase, a feature characteristic of osteoclasts, and were also responsive to calcitonin. Furthermore, they attached to mineralized bone particles and their membrane changed into a ruffled border at the zone of interaction with the bone matrix. We thus demonstrated that marrow endothelial-adipocytes may play a role in regulating the differentiation of myeloblasts into osteoclasts.

Key words: osteoclast precursors, stroma microenvironment, myeloblast cells, TRaP, ATPase

Osteoclasts are multinucleated cells which are found exclusively on bone surfaces. Their functions are coupled with active osteoblasts in maintaining the bone remodeling cycle. Osteoclasts are derived from mononuclear proliferating progenitors of hemopoietic origin. These cells differentiate and fuse to form multinucleated functional cells [Chambers, 1988; Gothlin and Ericsson 1976; Nijweide and deGrooth 1992; Mundy and Roodman 1989]. During the past decade, several attempts have been made to build a model for the investigation of osteoclast biology in vitro. Major involvement of marrow stromal cells was associated with the regulation of osteoclast differentiation. Cocultures of bone marrow or spleen cells with stromal cells were used to characterize the cell type responsible for osteoclast generation. The involvement of various osteoblastic cell lines in osteoclast regulation was demonstrated in cocultures in the presence of 1,25-$(OH)_2D_3$ [Suda et al., 1992; Takahashi et al., 1988; Udagawa et al., 1989; Zambonin-Zallone et al., 1984]. In other reports, osteoblastic cells failed to induce osteoclast formation in vitro [Chambers, 1992]. It has been suggested that osteoblastic cells secrete cytokines that are involved in the differentiation of preosteoclasts [Mundy, 1992]. We have previously shown that a marrow stromal osteoblast, the MBA-15 cell line, secretes M-CSF, GM-CSF, and IL-6 [Benayahu et al., 1992a]. These osteoblastic cells [Benayahu et al., 1992a] as well as others [Horowitz, 1993] secrete cytokines that may play a role in the differentiation of myeloid cells designated to form osteoclasts. In our studies, marrow osteoblastic MBA-15 cells were analyzed for their ability to induce a supportive hemopoietic inductive microenvironment (HIM), both in vitro [Benayahu et al., 1992a] and in vivo [Benayahu et al., 1992b, 1994]. We have shown that MBA-15 cells formed bone in heterotopic sites. However, the ossicle which had been formed could not support any recruitment of preosteoclasts or osteoclast differentiation [Benayahu et al., 1992b, 1994].

In many studies, cocultures of bone marrow cells with stromal cells were used to follow the process of differentiation into osteoclasts. The cellular composition of the marrow stroma is

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heterogenous and includes among others: fibroblasts, reticular cells, endothelial cells, adipocytes, and osteogenic cells. Owing to the fact that only some osteoblastic cells are capable of supporting osteoclast differentiation [Chambers, 1992] it is unclear which cell type is the main contributor to the control of the differentiation of osteoclasts. To address this question, we examined different stromal cell subtypes, members of the MBA series [Benayahu et al., 1991; Zipori et al., 1984, 1985], in coculture with a myeloblast cell line (M1). The introduction of M1 cells in coculture with the various stromal cell lines revealed unique relations with endothelial-adipocyte, the 14F1.1 cells. The latter is known to support hemopoietic stem cell growth and differentiation in vitro [Zipori and Lee, 1988; Zipori, 1992]. In the current culture system, we demonstrate the differentiation of M1 cells into osteoclasts induced by the 14F1.1 stromal cell line.

MATERIALS AND METHODS

Cell Lines

Marrow stromal cells (MBA series) included the MBA-1.1.1 fibroblastoid, MBA-2.1 fibroendothelial-like, MBA-15 osteoblastic, and 14F1.1 endothelial-adipocyte cell lines (a numeral to the right of the decimal point denotes a clonal cell line) [Benayahu et al., 1989, 1991; Zipori et al., 1984, 1985]. The myeloblastic cell line (M1) was spontaneously raised in SL mouse [Ichikawa, 1969]. Cells were maintained in Dulbecco's modified eagle medium (DMEM) high glucose (Bet-Haemek, Israel) supplemented with 10% fetal calf serum (FCS) or 10% horse serum (HS) (Bio-Lab, Israel), as indicated for each set of experiments and were incubated at 37°C in an atmosphere of 10% CO_2 in air.

Coculture Systems

MBA cells 1×10^5 were seeded in culture dishes (Falcon, New Jersey) grown to confluence and used as adherent layers. M1 cells were seeded on top of the adherent cell layer as specifically detailed for each experiment.

Adhesion of M1 Cell

M1 cells were loaded with 3×10^4 CPM of $^{51}Cr/10^4$ cells, washed, and seeded on stromal cell layers for 30 min. ^{51}Cr labeling of M1 cells and the adhesion assay were done using a method previously described [Juneja et al., 1992].

Colorimetric Assay for Cell Growth

Cell growth was measured in 96-well plates, using a colorimetric method [Mosmann, 1983]. From a stock solution (5 mg/ml) of (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide; (MTT) (Sigma, St. Louis, MO), 10 μl was added to assayed wells. Following 3 h of incubation at 37°C, acid isopropanol was added, mixed to dissolve the dark blue crystals, and read in a kinetic microplate reader (Molecular Devices Corp., Palo Alto, CA), using a test wavelength of 550 nm and a reference wavelength of 650 nm.

Flow Cytometric Analysis

M1 cells grown in control culture or from cocultures with 14F1.1 cells were incubated with αPgp-1 (CD-44) hybridoma supernatant or αMAC-1 antibodies for 90 min on ice with occasional agitation. The cells were rinsed in phosphate-buffered saline (PBS) and then incubated with goat anti-mouse FITC conjugated secondary antibody (Zymed, California). After 30 min, the cells were washed in PBS and a sample of labeled cells was analyzed using a Beckton Dickenson FACS.

Radioimmunoassay for cAMP

Cells were incubated in DMEM containing 5×10^{-4}M isobutyl methyl xanthine (IBMX) and 0.5% bovine serum albumin (BSA) for 15 min in 37°C and were then resuspended in the same medium with or without calcitonin (100 ng/ml) (Sigma, St. Louis, MO) for an additional 10 min. Following the hormone exposure, perchloric acid was added for distruction of the cells and then neutralized with potassium bicarbonate and this was followed by acetylation of the samples. cAMP was quantitated using radioimmunoassay (RIA) according to Harper and Brooker [1975]. For RIA we used polyclonal αcAMP antibody (Bio-Makor, Israel) and [^{125}I]-cAMP (NEN, England). Protein A (Bio-Makor, Israel) was used to separate the bound from the free ligand.

Histochemistry

M1 cells were collected from cocultures with 14F1.1 cells and compared to control M1 cells grown without stroma. The cells were spun on glass coverslips in a Sorvall cytospin apparatus, air dried, and kept at −20°C for application of the different cytochemical stains. Tartrate-

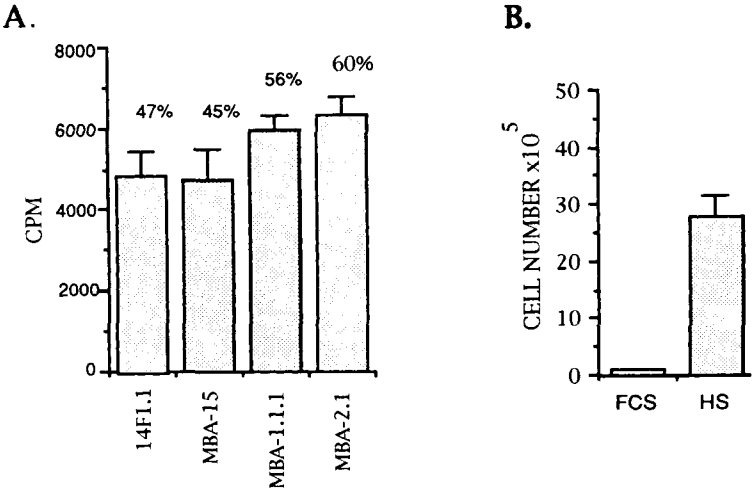

Fig. 1. Coculture of M1 myeloblast cells with stromal cells adherent layer. **A:** Adhesion of M1 cells loaded with ^{51}Cr (as described in Materials and Methods) and seeded on MBA stroma cell layers for 30 min. Quantitation of ^{51}Cr is presented as mean ± SD. **B:** Proliferation of M1 cells seeded on 10^3/well cocultured with 14F1.1 adherent cells in DMEM supplemented with either 10% FCS or 10% HS. Following 5 days of incubation, the number of cells was determined and expressed as mean ± SD.

resistance acid phosphatase (TRaP) staining was performed according to the instructions for use of the Sigma kit (386-1). Tartrate-resistance ATPase (TrATPase) was performed according to the method described by Andersson and Marks [1989].

Transmission Electron Microscopy (TEM)

Control M1 cells or M1 cells from cocultures with 14F1.1 stroma cells were washed three times with PBS and fixed with Karnovsky's fixative for 24 h at 4°C, after which they were washed with cocodylate buffer. Postfixation was performed in 1% OsO_4 in cocodylate for 1 h at room temperature followed by uranyl acetate for 1 h. After dehydration in graded ethanol, the samples were embedded in Epon 812. Thick sections were stained in toluidine blue for light microscopy (LM). Ultrathin sections were cut with a diamond knife on a Reichert ultramicrotome and stained with lead citrate and uranyl acetate were examined under a Philips EM 201 at 80 kV.

RESULTS

Adhesion and Proliferation of Myeloblast Cells in Coculture With Stromal Cells

The differentiation pathway of progenitor cells into osteoclasts requires interactions with stromal cells [Suda et al., 1992]. Our study was conducted to determine whether myeloblast (M1) cells respond to different marrow stromal cell lines. We first tested the ability of M1 cells to adhere to various stromal cells. ^{51}Cr-labeled M1 cells were cocultured onto MBA-1.1.1, MBA-2.1, MBA-15, and 14F1.1 adherent stromal cells. M1 cells attached equally well to all stromal cells (Fig. 1A).

We further studied the proliferation of M1 cells with cocultured stroma. Among the various stromal cell lines, only the 14F1.1 adherent layer was able to support long-term proliferation of M1 cells. Whereas poor growth was measured in the presence of FCS, M1 cells grew well when the cocultures were supplemented with HS (Fig. 1B, Table I). M1 cells usually grow in suspension, they are blast-like cells with a high nuclear/cytoplasm ratio (Fig. 2A). When cocultured with adherent 14F1.1 cells, close interactions between the two cell types were observed. The M1 cells attached to the 14F1.1 adherent layer (Figs. 1A, 2B) and the findings of phase contrast microscopy suggested that they were located underneath the stromal layer. M1 cells formed distinct colonies reminiscente of "cobblestone areas" (Fig. 2C). They proliferated mostly under the stromal layer (Fig. 3) when the cultures were supplemented with HS. In the latter cocultured M1 cells were accumulating in much greater numbers compared to those supplemented with FCS (Figs. 1B, 3).

Differentiation of Myeloblast Cells in Coculture With 14F1.1 Stroma Cells

The cellular interactions between M1 cells and the 14F1.1 stromal cells were examined by

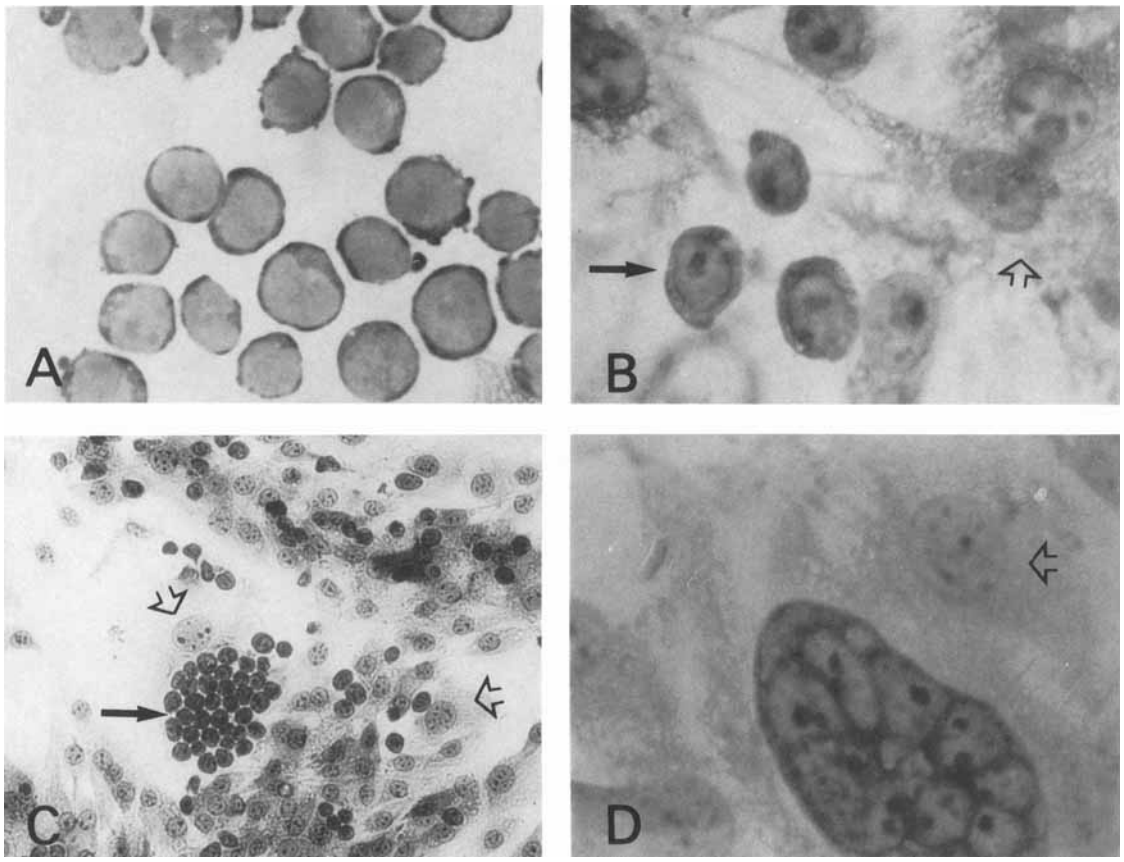

Fig. 2. Morphological appearance of M1 cells and of cocultures of M1 cells with the 14F1.1 adherent layer. **A:** Control M1 cells grown in suspension. **B:** M1 cells (arrow) attach to the adherent layer and **C** forming cobblestone-like areas (arrow). **D:** Multinucleated cells. The open arrow indicates a 14F1.1 stromal cell. The cultures were stained with May-Grunwald Giemsa (A, B, D, × 250; C, × 50).

TABLE I. Growth of M1 Cells in Coculture With Various Stromal Cell Lines*

	M1 proliferation	
Cell lines	FCS	HS
MBA-1.1.1.	–	–
MBA-2.1	–	–
MBA-15	–	–
14F1.1	+	+++

*M1 cells (10^3/well) were seeded onto stromal cell cultures supplemented with either 10% FCS or HS. –, no proliferation; +, < 1 × 10^5 cells; +++, > 3 × 10^5 cells. The growth medium was replaced weekly, the cocultures were monitored for the proliferating M1 cells at 5 days culture as described in Figures 1B and 3. Cocultures were maintained up to 3 weeks.

light microscopy of fixed cultures (Fig. 2B–D) and by phase contrast microscopy (Fig. 4). The distinct M1 cell foci resembled "cobblestone areas" (Fig. 2C). Two types of association were observed by TEM. Some M1 cells were surrounded by cytoplasmic projection of 14F1.1

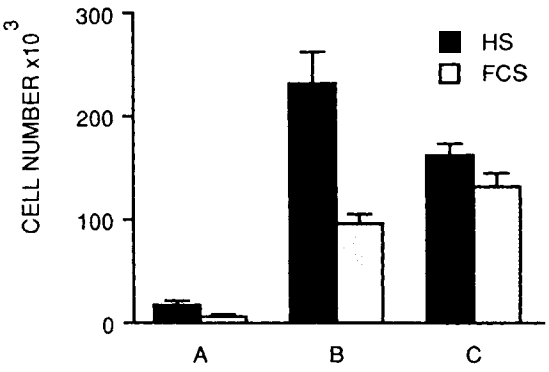

Fig. 3. Proliferation of M1 cells in the presence and absence of 14F1.1 cells. M1 cells were seeded on a 14F1.1 cell layer and supplemented with 10% FCS or 10% HS. Five days later, the numbers of cells in the liquid phase above the stroma layer (**A**), cells within the stroma layer (**B**), and the number of cells growing in control cultures without stroma (**C**) were counted. The M1 cell proliferating within the stromal layer were enumarated following trypsinization of the cocultures fine pipetation that released the attached M1 cell from the adherent layer and readhering the 14F1.1 stroma following dilution. The results are expressed as number of M1 cells per plate ± SD.

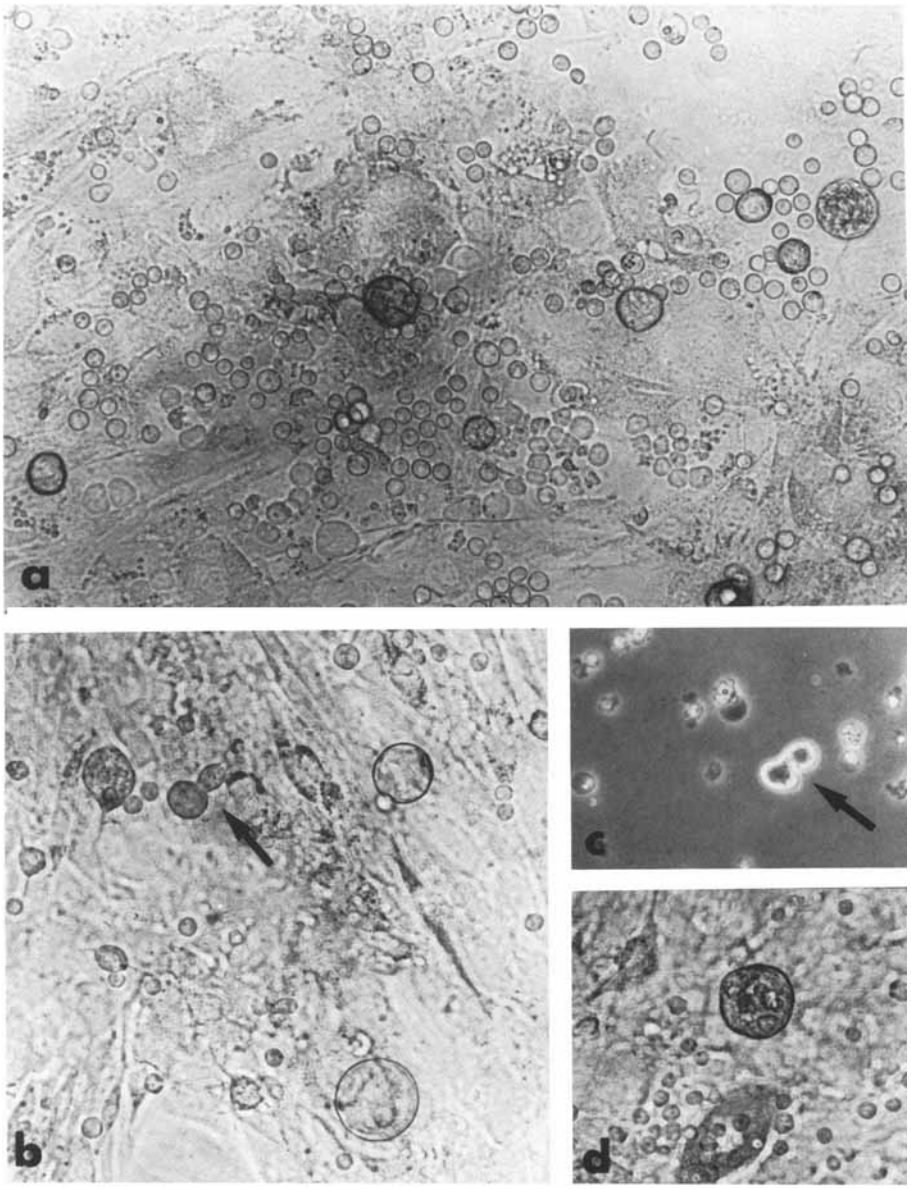

Fig. 4. Phase contrast micrographs in which proliferating M1 cells cocultured with adherent 14F1.1 cells are seen. The sizes of the M1 cells varied and large multinucleated cells can be distinguished from small mononuclear cells (**a–d**). The cellular interactions possibly leading to cell fusion (arrow) result in large multinucleated cells (b,c). ×50.

cells (Fig. 5A,B). In other cases we observed cellular cytoplasmic projections from M1 cells "invaginating" 14F1.1 cells and some of the M1 cells attached among themselves through cytoplasmic pseudosoms (Fig. 5B,C).

M1 cells in coculture with 14F1.1 cells had a variable cell size. Small mononuclear and variable sizes of multinucleated cells were observed (Figs. 2, 4). Mononuclear cells in doublets, which are possible participants in the fusion process to multinuclear cells, were also detected (Fig. 4b,c). The multinucleated cells which had formed were found either adhering to the 14F1.1 layer (Fig. 2D) or in the liquid phase of the cultures (Fig. 4a,b,d).

As indicated by FACS analysis, the homing receptor CD-44 (Pgp-1) was expressed by M1 cells (Fig. 6). This surface receptor is capable of taking part in the cell-to-cell interactions between the M1 cells and the 14F1.1 stroma layer. M1 cells failed to express MAC-1, a marker of myeloid progenitors. When these cells were

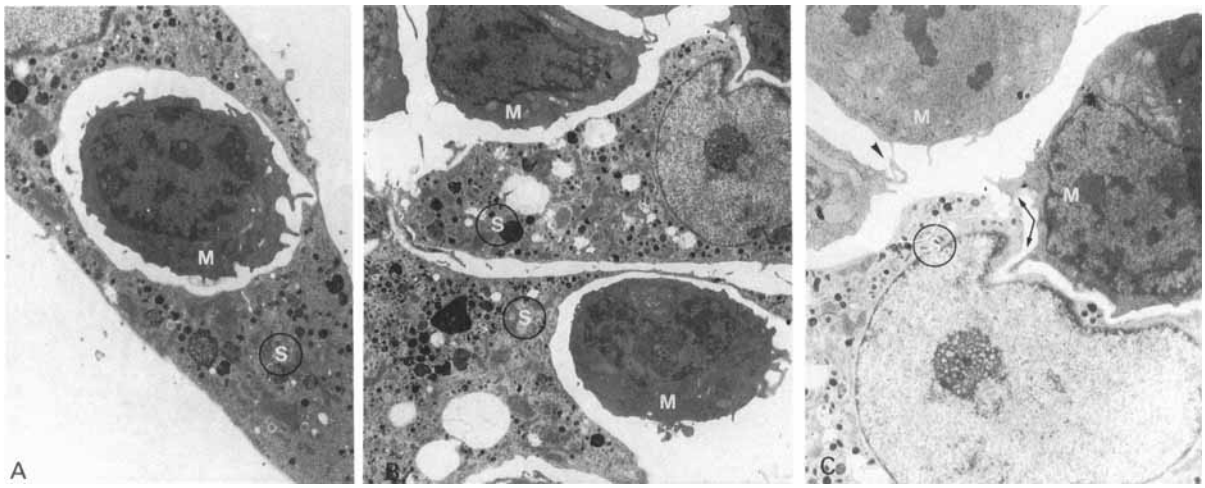

Fig. 5. TEM analysis of M1 cell (M) associated with the stroma layer of 14F1.1 cells (S). M1 cells were cocultured without stroma for 5 days, and the micrographs show M1 cells surrounded by 14F1.1 cell projection, with the M1 cells being "engulfed" by 14F1.1 nesting cells (**A**, ×4,300). Projections from an M1 cell "invaginating" the nesting 14F1.1 cells (arrow) or of the M1 cells among themselves (arrowhead) are indicated (**B**, ×2,800; **C**, ×4,300).

grown in association with 14F1.1 cells for 14 days, they became MAC-1 positive (Fig. 6). Since this may imply that M1 cells differentiated upon coculture with 14F1.1 cells, we further studied this possibility by examining their enzymatic activity. M1 cells are negative for the enzymatic activities of TRaP and ATPase. Upon coculture with 14F1.1 cells, both mononuclear and multinuclear M1 cells were positive for TRaP. The TRaP-positive cells differed in the intensity of staining (Fig. 7). These cells also expressed ATPase activity (Fig. 8). These features suggest that the cells had acquired preosteoclastic properties. To further examine this possibility, nonadherent cells were collected and were seeded with mineralized bone particles. Light microscope analysis revealed M1 cells that were attached to the bone particles (Fig. 9a,b). The latter cells were positive to TRaP (Fig. 7D) and ATPase (Fig. 8b). Ultrastructural appearance was analyzed by TEM. Multinucleated cell was presented (Fig. 10a) and cells with lysosomal vacuoles (Fig. 10b). The M1 cells attached to bone particle and the cellular reaction produce a complex of membranal change at the bone-surface interface. The membranal change acquired a complex of deeply infolding finger-like plasma membranes that are associated with bone matrix substrutum. The external plasma membrane formed the ruffled border zone (Fig. 10c,d).

The response of M1 cells to calcitonin was monitored following 14 days of coculture with

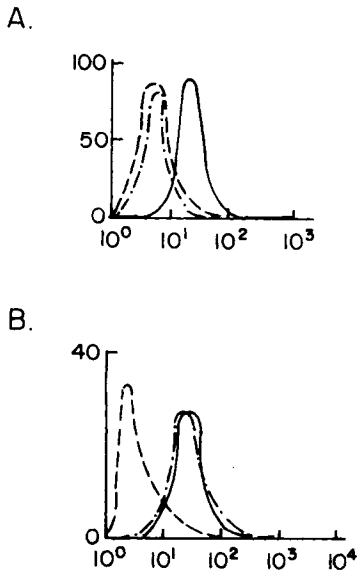

Fig. 6. FACS analysis of expression of CD44 (Pgp-1) or MAC-1 by M1 cells in control cultures (**A**) and following coculture with 14F1.1 (**B**). The interrupted line (- - -) represents negative control (IgG); the peaks represent fluorescence obtained with MoAb directed against the CD44 (solid line) or against the surface antigen MAC-1 (dotted line).

14F1.1 cells. Nonadherent M1 cells were collected and exposed to calcitonin. Using RIA assay, the intracellular cAMP monitored was 61.66 ± 4.7 fmole/10^5 in cocultured cells as compared to 9 ± 5.09 fmole/10^5 in control cells, i.e., a 7-fold increase.

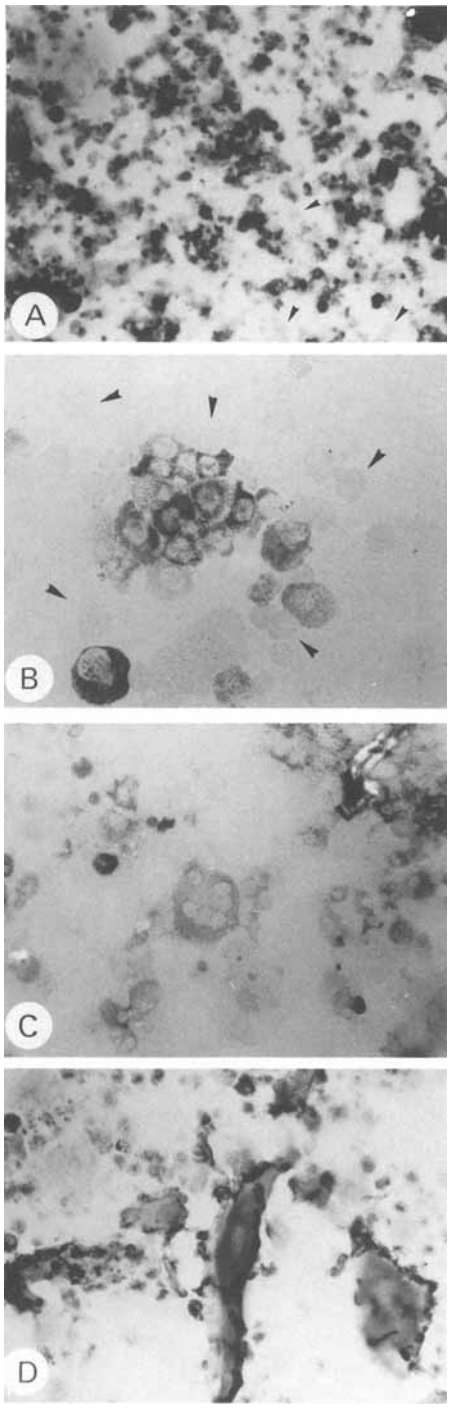

Fig. 7. TRaP staining of M1 cells cocultured with 14F1.1 stroma for three weeks. The heterogeneous population included TRaP-positive mononuclear precursors and multinucleated cells. (**A–C**) M1 cells which were TRaP-activity negative are also visible (B, arrowheads). **D:** Cells attached on bone particles are also found to be TRaP positive. (A, D, ×50; B,C, ×200).

DISCUSSION

One of the most striking events in the developmental biology of bone is the establishment of hemopoiesis within the medullary cavity. The topographical association between hemopoietic tissue and bone implies mutual dependence and cross-regulation. The hemopoietic microenvironment is crucial for the regulation of hemopoietic growth and differentiation. This microenvironment is also important for bone development and involves a range of cellular interactions. The developmental cascade for bone-hemopoietic organ formation was demonstrated in ectopic sites in vivo. It was demonstrated by implantation of a demineralized bone/tooth matrix that induced cartilage and bone formation in heterotopic sites [Benayahu et al., 1992b, 1994; Wientroub et al., 1982]. The cascade of events in ossicles induced by implanted matrix include initial proliferation of mesenchymal cells which is followed by cartilage model formation. The cartilage mineralizes and then becomes hyperotrophic, eventually being gradually replaced by bone. The newly formed bone then continues to become remodeled and is repopulated with hemopoietic cells. Since osteoclasts are derived from a hemopoietic origin, the observation that ectopic ossicles become populated by hemopoietic cells indicates that bone tissue creates a suitable HIM which is also necessary for osteoclast development. Osteoblasts were recognized as being necessary for osteoclast differentiation and maturation. Their role in the process of osteoclast differentiation was shown to be dependent in vitro on $1,25(OH)_2D_3$ [Suda et al., 1992]. Other studies did not support the notion that osteoblasts are major regulators of osteoclast differentiation [Chambers, 1992]. Also, the role of osteoblasts in homing or recruitment of osteoclast precursor cells in vivo has not yet been defined. The findings of our in vitro studies and in vivo experiments demonstrated an impairment of osteoclastogenesis in vivo occurring when heterotopic bone was formed by marrow stroma osteoblasts [Benayahu et al., 1922b, 1994].

The present study was aimed at examining the effects of stroma cells on osteoclast differentiation. A coculture system of stromal cells (14F1.1) with myeloblastic precursors was established and served as a supportive microenvironment for such differentiation. The cloned cell line, 14F1.1 supports hemopoiesis in long-term

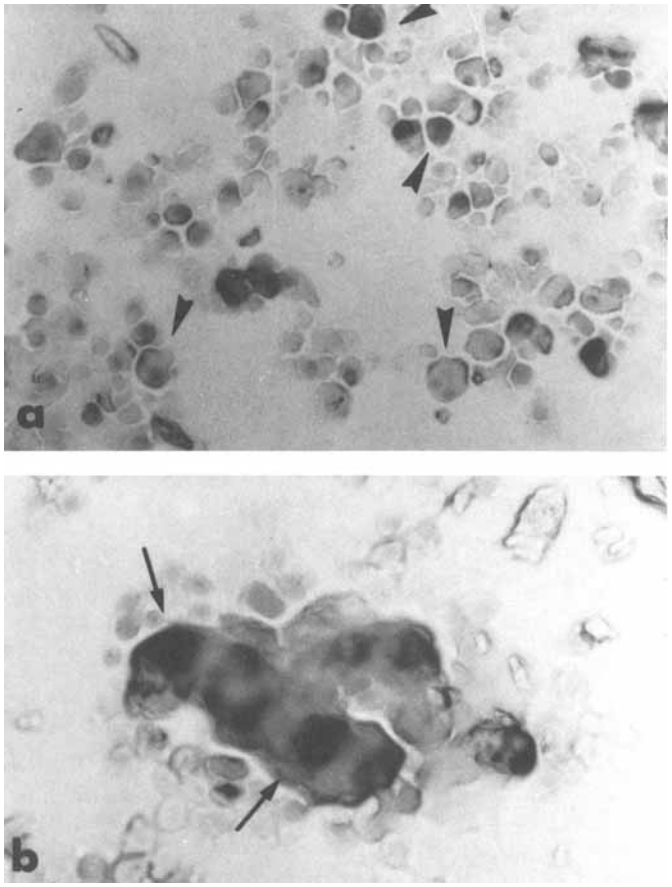

Fig. 8. ATPase staining of M1 cells cocultured with 14F1.1 stroma for three weeks. Positive cells are visible (arrowheads) (**a**, ×50). Cells attached onto bone particles are ATPase positive (arrows) (**b**, ×50).

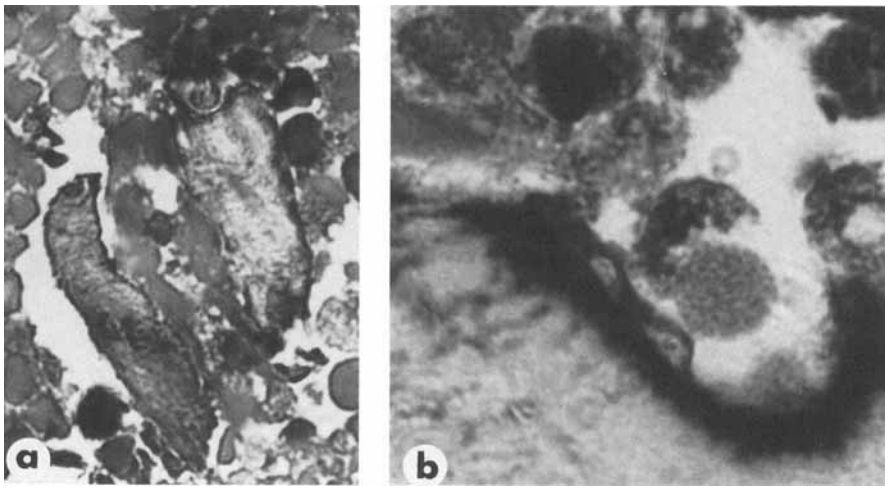

Fig. 9. Light microscopy analysis of cells which are seen to be attached onto the bone particle surface. A 5 μm thick section stained with toluidine blue is shown (**a**, ×50; **b**, ×200).

cultures [Zipori and Lee, 1988; Zipori, 1992]. In the current study, 14F1.1 cells were found to be supportive for the induction of osteoclast differentiation from the myeloblastic cell line, M1. The interactions between marrow stromal cells and preosteoclasts may initially involve cell surface recognition and direct localization to the right microenvironment in vivo. M1 cells were

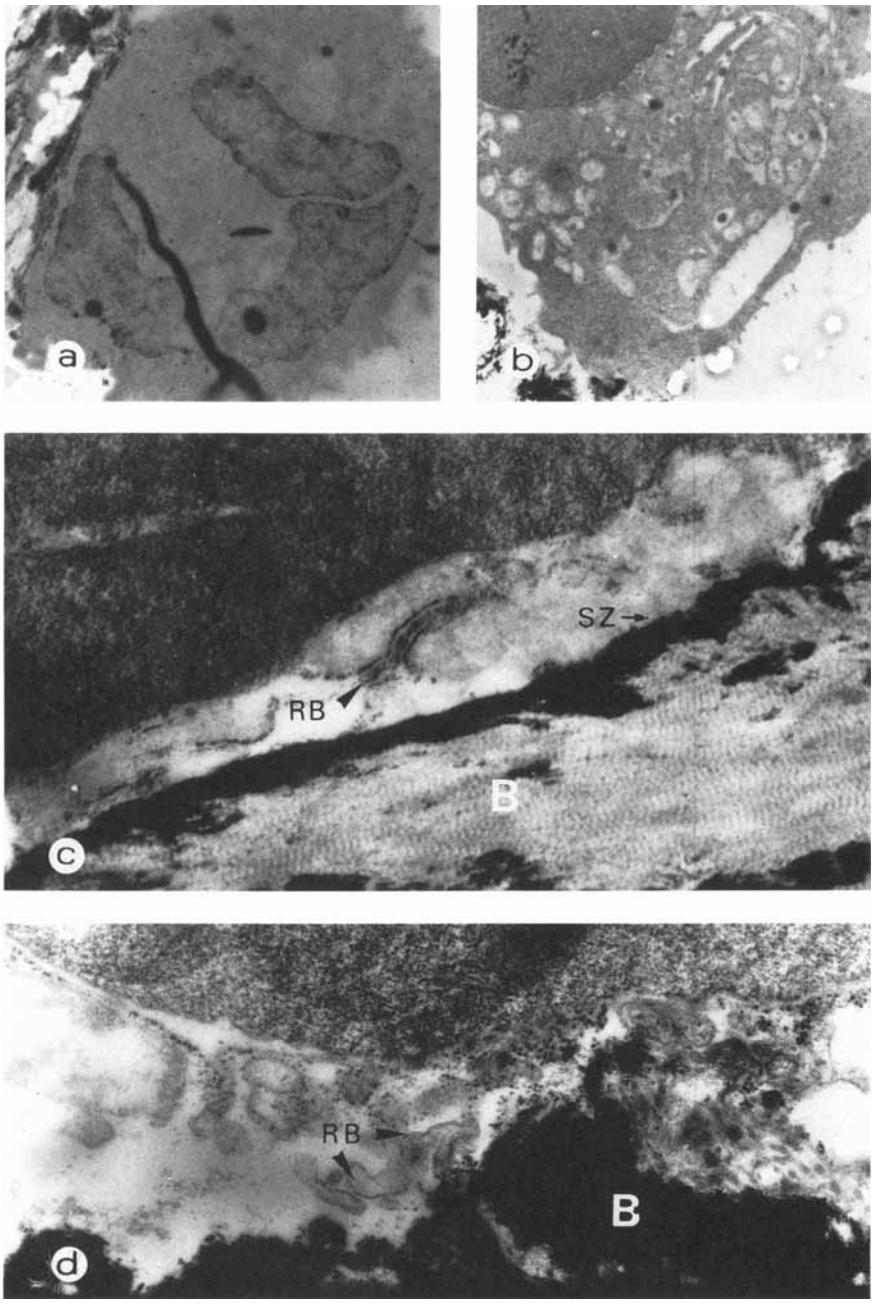

Fig. 10. TEM analysis of (**a**) a multinucleated cell and (**b**) a cell with vacuoles and lysosomes. **c,d:** At the contact region between the M1 cell cocultured with bone particle (B), the plasma membrane acquired changes. Towards the extracellular space delineation of deeply infolding finger-like plasma membranes, the ruffled border membrane (RB) (arrowhead) along the bone matrix, adjacent to the sealing zone (SZ) was demonstrated (×3,400).

shown to exhibit high levels of the cell surface receptor CD-44, which may serve as a mediator in the cellular interactions with stromal 14F1.1 cells. CD-44, which is a widely expressed molecule, is involved in functions ranging from fibroblast adhesion to collagen and lymphocyte homing to endothelial cells. Experiments have been conducted which show that CD-44 molecules can bind hyaloronic acid, fibronectin, and collagen III and VI [Arrufo et al., 1990; Hyman et al., 1991; Lesley et al., 1990; Miyake et al., 1990]. Other cellular interaction with ECM components are modulated via integrin receptors [Clover et al., 1992, Horton and Davis, 1989].

In this study, the differentiation of M1 cells induced by 14F1.1 stromal cells was measured by morphological criteria, enzymatic activity, and calcitonin response. A correlation between lysosomal activity and osteoclast differentiation has been demonstrated, i.e., osteoclasts associated with high TRaP activity [Minkin, 1982], ATPase [Andersson and Marks, 1989; Fukushima et al., 1991], and (Na+/K+)ATPase [Baron et al., 1986a] activities. Two types of acid phosphatases have been identified in bone tissue, on the basis of sensitivity to tartrate and have been employed as a histochemical marker to identify osteoclasts and preosteoclasts [Akisaka et al., 1989; Baron et al., 1986b; Chappard et al., 1983; Cole and Walters 1987; Minkin, 1982; Tanaka et al., 1987; Van de Wijngaert and Burger, 1986]. The appearance of TRaP-positive mononuclear cells is characteristic of osteoclast precursor cells before they fuse into multinucleated cells [Van der Pluijm et al., 1991]. Identifing these precursors may be important to enable the study of their cellular differentiation pathway under regulation of local growth factors and systemic hormones. Whereas M1 cells do not express acid phosphatase activity, this enzyme was demonstrated histochemically following coculture of M1 with the 14F1.1 adherent layer: mononuclear precursor and multinuclear cells were positive to TRaP and ATPase staining. The mononuclear TRaP-positive cell cytodifferentiation is known to be influenced by specific matrix components [Krukowski and Khan, 1982; Webber et al., 1990; Osdoby et al., 1988]. In our study, upon interaction with mineralized bone particles, the membranal portion of M1 cells underwent morphological changes, producing a ruffled border at the site of interaction with bone matrix. This unique morphological change is a prerequisite for osteoclastic function.

Calciotropic hormones play an important role as humoral regulators for osteoclast differentiation. In the presence of $1\alpha,25(OH)_2D_3$, a subpopulation of marrow mononuclear cells form multinucleated cells with osteoclast characteristics [Roodman et al., 1985]. Parathyroid hormone (PTH) acts as potent stimulators for new osteoclast formation. Calcitonin is known to be involved in regulating the processes of bone resorption and bone remodeling and osteoclasts possess high levels of calcitonin receptors [Warshawsky et al., 1980]. The cocultured M1 cells were indeed highly responsive to calcitonin as measured by an increase in cellular cAMP.

Taken together, the data presented above inducated that the 14F1.1 stroma cells induce the differentiation of M1 myeloblasts into cells that aquired osteoclast properties. This process did not require induction by any external agent such as $1,25(OH)_2D_3$.

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