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A Novel Culture System to Generate Osteoclasts and Bone Resorption Using Porcine Bone Marrow Cells: Role of M-CSF

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A novel osteoclast generation and bone resorption assay system is described in which enhanced osteoclastic generation and bone resorption is induced in porcine bone marrow cell cultures cultured in low-serum medium supplemented with fibroblastic cell (L929) conditioned medium (CM). Numerous osteoclasts, which could be identified by TRAP staining and specific labelling with 121F antibody, were generated in a time-dependent and L929-CM concentration-dependent fashion. A specific antibody against murine M-CSF/CSF-1 abolished osteoclast formation indicating that M-CSF is the essential component of the L929-CM driven osteoclast generation. Culturing on devitalized bone slices resulted in extensive osteoclast-mediated resorption as visualized microscopically. After 16 days in culture, pratically the entire bone slice surface was excavated by the osteoclastic cells. Bone resorption could be monitored with time using a novel enzyme-linked immunoassay measuring type I collagen N-telopeptides in culture supernatants. Release of collagen fragments from the slices was paralleled by osteoclastic secretion of TRAP. Salmon calcitonin significantly inhibited collagen fragment and TRAP release. 1,25-Dihydroxyvitamin D_3 greatly promoted osteoclast generation and subsequent bone resorption, but its presence was not essential for this process to occur.

Osteoclasts, the multinucleated giant cells primarily responsible for resorption of bone are derived from hematopoietic progenitor cells which are closely related to the mononuclear phagocyte family (1). Macrophage colony-stimulating factor (M-CSF/CSF-1), the hematopoietic growth factor involved in mononuclear phagocyte growth and function, has also been recognised to be indispensable for proliferation, differentiation of osteoclast progenitors and survival of multinucleated osteoclasts (for reviews: 2,3). M-CSF is produced by osteogenic-stromal cells and released into the extracellular bone environment or presented in a membrane- or matrix-bound form (4,5). The receptor for M-CSF is encoded by the proto-oncogene *c-fms*, and is specifically expressed on the cells of the mononuclear phagocyte family including cells belonging to the osteoclast lineage (6,7). Bone marrow cells are generally used as source of osteoclast progenitors to study regulation of osteoclast differentiation. Stromal cells or osteoblastic cells are considered to be required to provide an inductive layer to enable osteoclast differentiation necessarily through cell-cell contact; in addition the presence of $1,25(OH)_2D_3$ is necessary to sustain osteoclast induction, especially in the widely used rodent models (3), but it is not clear whether that is true for primate or avian species (1). Here, we describe an alternative mammalian culture system using porcine bone marrow cells cultured in the presence of fibroblastic (L929) conditioned medium resulting in induction of numerous osteoclasts with extensive bone resorbing capacity.

MATERIALS AND METHODS

Cell cultures. L929 is a murine fibroblastic cell line used as a source of M-CSF/CSF-1 (8). L929 cells were cultured in DMEM with 10% fetal bovine serum (FBS) until confluent. Cell-free conditioned medium (CM) was harvested and stored frozen until use. A specific antibody directed against murine M-CSF, a generous gift from Prof R Stanley (9) was used to determine the role of biological M-CSF activity in the L929-CM.

Newborn pigs (white/landrace; 1-2 days' old) were euthanized by intracardiac sodium barbitone injection. A maximum of 1 hour thereafter, long bones from the hindlimbs were dissected, rinsed with 70% ethanol, and transversely cut along one of the epiphyses with a scapel. Bone marrow cells were scraped from the bones into sterile phosphate buffered salt solution, and the trabecular bone particles were removed by gravity sedimentation. The marrow cells were suspended in MEM supplemented with 10% FBS and precultured for 4 hours in a 5% CO2 humidified incubator. Nonadherent cells were collected, resuspended in αMEM/2%FBS, counted and transferred in 96-multiwell dishes (2.10^5 nucleated cells per well). The final culture medium was composed of αMEM containing 2-4% FBS and 25% (v/

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v) L929-CM with or without 10^{-8} M $1,25(OH)_2D_3$. Half of the medium was changed every 2-3 days.

Characterization of osteoclasts. Tartrate resistant acid phosphatase (TRAP) was determined cytochemically using a Sigma kit with naphtol AS BI phosphatase as substrate and Fast Garnet as coupler in the presence of 10mM sodium tartrate (15 min. at 37°C) following fixation of adherent cells with citrate-buffered formalin for 90 sec. TRAP was measured biochemically by incubating the cell layer in 0.1M sodium acetate solution, pH 5.0, containing 2 mg/ml para-nitrophenol phosphate (PNPP) and 0.1% Triton X-100. The reaction was stopped after 1h with 0.1M NaOH, and the production of PNP was determined by an automatic multiwell reader at 405 nm. Protein content was determined using a modified Lowry assay for 96-well plates with albumin as standard.

Osteoclastic cells were further characterized by immunolabelling with a specific anti-osteoclast antibody 121F which was kindly provided by Prof P Osdoby (10). After fixation, cells were blocked with PBS/0.4% BSA for 30 min at room temperature followed by labelling with 121F anitbody for 90 min. (1:200 in PBS/0.4%BSA). Antibody visualization was performed with anti-mouse IgG conjugated with FITC (Sigma; 1:100 in PBS/0.4% BSA) labelling for 1 h.; immunofluorescence was examined using a Zeiss microscope.

Bone resorption. In bone resorption experiments the marrow cells were seeded on ivory or porcine cortical bone slices in Iscove's medium, pH 6.9, with the similar supplements as above. Medium was harvested every 2-3 days, and replaced with fresh medium. After culture, the cells were removed from the slices by several rinses with distilled water. Dried slices were used for examination by a Philips scanning electron microscope. In addition, the slices were viewed and measured with a contrast reflection microscope coupled to a Leitz Quantimet image analysis system. Bone resorption was monitored quantitatively by analysing the release of type I collagen N-telopeptide fragments into the CM with a novel ELISA kit (Metra Biosystems, Mountain View,CA) based on a monoclonal antibody reacting specifically with a sequence in the N-terminal telopeptide of the α2(I) chain (J. Ju, V. Kung, V. Liu, unpublished observations). In addition, culture supernatants were analysed for TRAP activity using a biochemical assay with 2 mg/ml para-nitrophenol phosphate (PNPP) as substrate in 0.1M sodium acetate solution, pH 5.0. The reaction was stopped after 2 hours with NaOH, and the production of PNP was determined by an automatic multiwell reader at 405 nm.

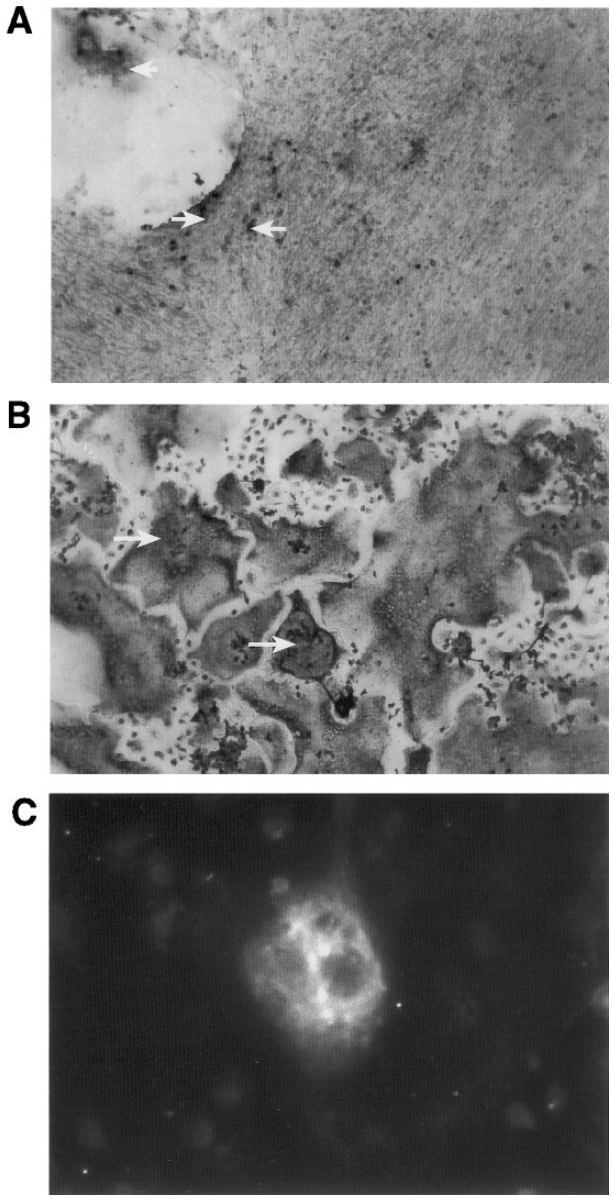

FIG. 1. Osteoclast-like cells formed in porcine bone marrow cultures supplemented with 10^{-8} M $1,25(OH)_2D_3$. A. Control culture after 9 days without L929-CM stained for TRAP. Note that the osteoclast-like cells (arrows) associated with the stromal cell layer. (×500) B. Numerous TRAP positive osteoclast-like cells are dominating in parallel culture which was supplemented with 20% L929-CM. Arrows point to TRAP-postive osteoclast-like cells (×500) C. Positive immuno-fluorescence labelling of osteoclast-like cell with 121F antibody (×1,000).

RESULTS

Numerous osteoclast-like cells were generated from porcine bone marrow cells cultured in the presence of L929-CM after 7 days of culture in low-serum media. The cells featured positive TRAP staining as well as labelling with 121F antibody (Fig. 1). The addition of $1,25(OH)_2D_3$ clearly promoted osteoclast differentiation, but its presence was not essential for osteoclast formation. L929-CM dose-dependently inceased biochemical specific TRAP activity (Fig. 2). M-CSF was the growth factor responsible for the L929-CM bioactivity as a specific antibody against murine M-CSF completely abolised L929-CM driven osteoclast generation (Fig. 3). The osteoclasts were able to resorb bone when cultured upon dentine or cortical slices. Microscopic examination showed extensive resorption of the slice surface (Fig. 4). After 16 days, the resorbed surface amounted to 63.3 ± 11.1% (mean ± SD; n=3) of the dentin slice surface, which increased to 99.9 ± 0.4% (mean ± SD; n=4) in the presence of $1,25(OH)_2D_3$. Bone resorption could accurately and quantitavely be monitored by analysing the release of collagen type I fragments into the medium (cumulative release shown in Fig. 5). This was paralleled by the production and secretion of TRAP into the medium (Fig. 5). Calculating collagen fragment/TRAP release on a daily basis showed that bone resorption in the presence of $1,25(OH)_2D_3$ caused an early onset of osteoclastic re-

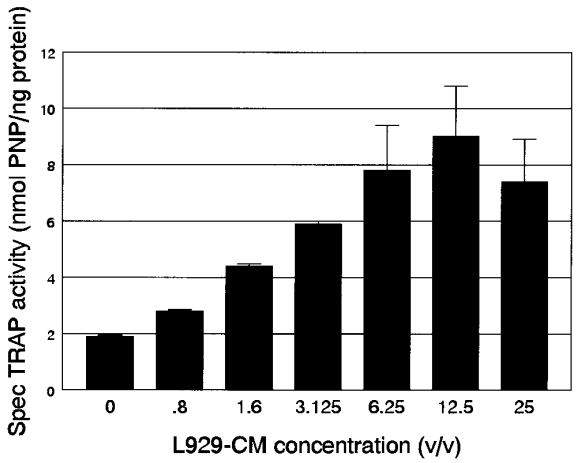

FIG. 2. Dose-dependent stimulation of osteoclast formation from porcine bone marrow cells by L929-CM in the presence of $1,25(OH)_2D_3$ after 7 days as assessed by cellular biochemical TRAP activity. Mean ± SEM of 6 replicates.

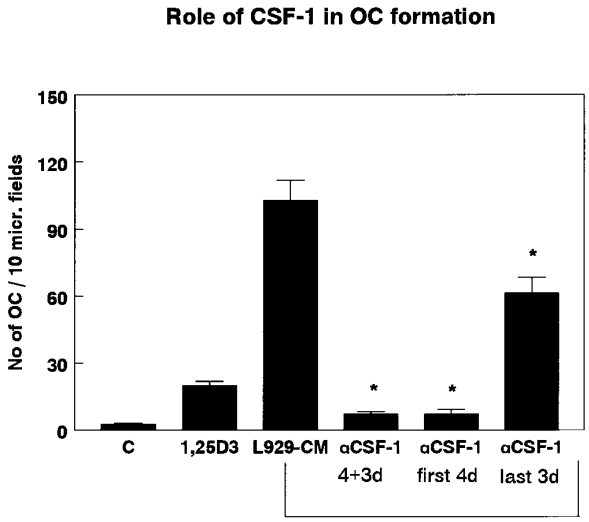

FIG. 3. Anti-M-CSF/CSF-1 antibody inhibits osteoclast formation in 7 days' total porcine marrow cultures. The number of TRAP-positive osteoclasts were counted in 10 microscopic fields per well (mean ± SEM of 6 replicates). The antibody dilution used (1/2,000) was adequate for full inhibition of L9-cell stimulated murine M-CFU growth in colony-forming assay using semi-solid medium. Note that incubation with the anti-M-CSF antibody during the first 4 days is sufficient for essentially complete abolition of osteoclast formation.

sorption which peaked after 14 days and gradually decreased thereafter, whereas cultures without $1,25(OH)_2D_3$ showed significant resorption after 7-10 days and reached $1,25(OH)_2D_3$-levels after 15-16 days. Treatment of L929-CM + $1,25(OH)_2D_3$ osteoclast cultures on ivory slices with 10^{-8} M salmon calcitonin for three days (from 16-19 days) significantly inhibited release of collagen fragments to 64.5 ± 7.4% (relative mean ± SD, n=4; $P<0.01$), and supernatant TRAP levels to 61.7 ± 18.5% (relative mean ± SD, $P<0.01$) of control levels, confirming that the resorption process was mediated by true osteoclasts.

DISCUSSION

This paper presents an easy and suitable system to study osteoclast differentiation using porcine bone marrow cells. L929-CM promoted formation of numerous osteoclasts resulting in a dramatic increase in the level of bone resorption. The essential component of L929-CM responsible for osteoclast induction was confirmed to be M-CSF as a specific anti-M-CSF antibody completely abolished osteoclast generation. Our approach using L929-CM complements a recent report by Sells Galvin et al. (11) who reported on the use of newborn porcine bone marrow cells to generate osteoclasts in culture, and is an ideal mammalian system, as an alternative to rodent and avian models, to study osteoclast differentiaton and function. The yield of osteoclast-like cells is not only extremely high, but the level of bone resorption obtained in our culture system greatly exceeds the levels reported in other studies us-

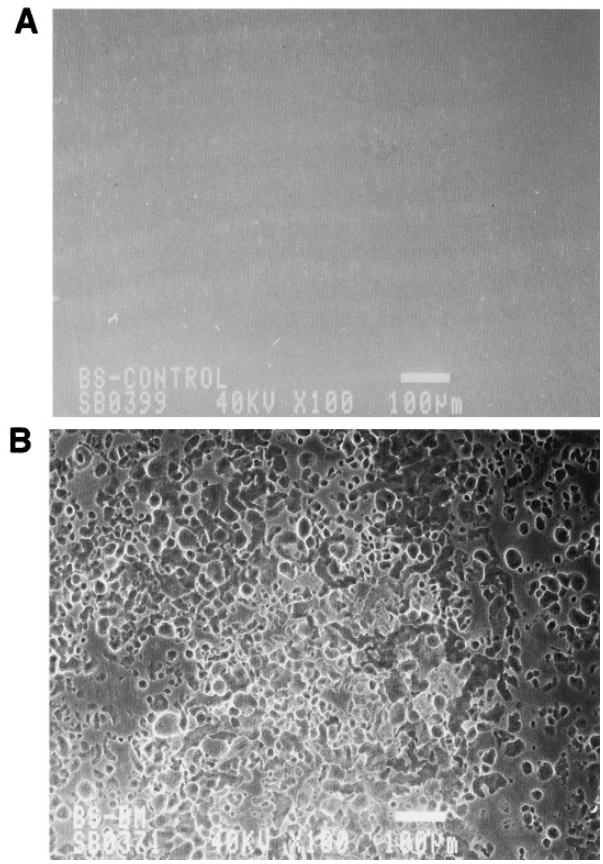

FIG. 4. SEM micrographs of ivory slices maintained in culture medium for 16 days. A. Control slice without cells; B. Slice cultures with bone marrow cells in presence of 20% L929-CM. Note the extensive excavation (resorption areas).

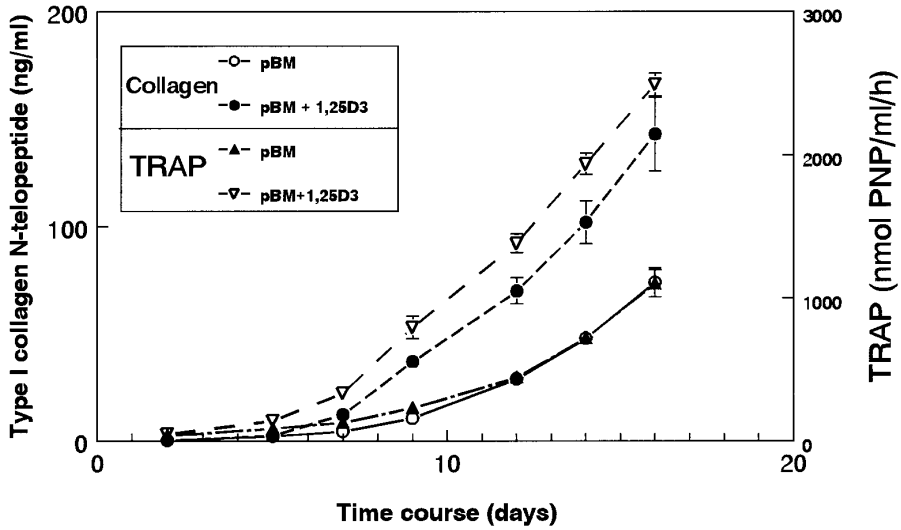

FIG. 5. Time course of resorption as monitored by analysis of collagen type I telopeptide fragment release from ivory and porcine cortical bone slices and osteoclastic TRAP secretion into culture supernatants from total porcine marrow cultures maintained in L929-medium (20%) +/− 10^{-8} M $1,25(OH)_2D_3$. Data are expressed as cumulative values (mean ± SEM for 4 replicate cultures).

ing isolated osteoclast-like cells or using osteoblast/stromal-cell supplemented marrow co-cultures or osteoclastic cell lines. It is generally known that in murine bone marrow cultures both the presence of $1,25(OH)_2D_3$ and direct cell contact with stromal-like cells are obligatory for osteoclast induction (1,3). Moreover, M-CSF appears to be indispensable for this process to occur (2,3). M-CSF is known to interact with many aspects of osteoclast biology, including progenitor proliferation and differentiation, survival and mobility of mature osteoclasts (2,3). However, conflicting data have been published as to the effects of exogenously administrated M-CSF on osteoclast formation in marrow cultures (12,13). M-CSF has also been reported to inhibit mature osteoclast resorptive activity (14). Nonetheless, supplementation of M-CSF was required for osteoclastic differentiation in murine marrow and human monocytic co-cultures with stromal and osteoblastic cells (15,16). M-CSF was used as a tool to generate (M-CSF-dependent) osteoclast-like cell lines from *H-2K^{b}tsA58* transgenic mice (17). Moreover, it has been reported recently that supplementation of human bone marrow cultures with recombinant M-CSF greatly promoted osteoclast differentiation and resorption (18). Shevde et al. (19) used L-cell CM in the basal medium to investigate regulation of osteoclastogenesis in murine marrow cultures, but effects on bone resorption were not mentioned in their studies. In our porcine cultures, conditions generated by the presence of L929-CM appeared to be ideal for sustained of osteoclast generation, as well as bone resorption. Bone resorption could easily be monitored by measuring collagen type I fragments released from the bone slices into the medium by collagenolysis using a novel ELISA method. A similar approach using the 'Crosslaps assay' was described previously as a reliable method to quantitatively follow bone resorption in culture (20). Collagen breakdown paralleled formation of resorption pits and release of biochemical TRAP activity into the medium, indicating that cellular release of TRAP is closely associated with the process of bone resorption.

In summary, we describe an extremely potent osteoclast generation model system using L929-CM as a tool in porcine bone marrow cell cultures. This assay system will facilitate further studies of osteoclast progenitor development and regulation of bone resorption.

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