Stromal Cell–Mediated Stimulation of Osteoclastogenesis (43711)

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Abstract. **In the bone marrow microenvironment, stromal cells or their products are known to regulate proliferation and differentiation of hematopoietic stem cells. The purpose of this investigation was to characterize stroma-mediated effects of differentiation-inducing factors on osteoclastogenesis in defined murine cultures. Hematopoietic progenitors (derived from long-term bone marrow cultures, LTBMCs) were cocultured with cloned stromal cell lines to demonstrate the indirect effects of various differentiation-inducing factors. Osteoclastogenesis was compared in three murine marrow systems (whole bone marrow, progenitors cultured alone, and cocultures of progenitors with stromal cell lines) by analysis of multinuclearity and tartrate-resistant acid phosphatase (TRAP) activity. The cultures were treated for two weeks with murine recombinant GM-CSF (5 U/ml), 1,25-dihydroxyvitamin D_3 (10^{-8} *M*), or parathyroid hormone (PTH, 10^{-8} *M*). In whole bone marrow cultures, osteoclast differentiation was stimulated by GM-CSF, PTH and 1,25-dihydroxyvitamin D_3. With progenitors alone, only GM-CSF promoted osteoclastogenesis. Each agent stimulated osteoclastogenesis in cocultures of progenitors with a stromal cell line (GBLneo^r). Thus, the coculture system is a partially defined model for whole bone marrow cultures. In contrast, progenitors that were cocultured with a stromal cell line derived from an osteopetrotic *op/op* mouse failed to differentiate in the presence of PTH or 1,25-dihydroxyvitamin D_3. These results indicate that stimulation of osteoclastogenesis by PTH or 1,25-dihydroxyvitamin D_3 is mediated indirectly through factors present in normal marrow stromal cells and that an osteopetrotic stromal cell line failed to support differentiation.** [P.S.E.B.M. 1994, Vol 205]

Osteoclasts are large multinucleated cells that are responsible for the resorption of bone during development, growth, and remodeling. These cells are also involved in pathologic destruction of bone in conditions like osteoporosis, rheumatoid arthritis, and periodontal diseases. On the other hand, in osteopetrosis, decreased osteoclastic bone resorption results in sclerotic bone with a spectrum of clinical consequences. Evidence has accumulated from several experimental systems including chicken-quail chimera experiments (1), parabiotic studies (2, 3) and marrow transplantation studies (4, 5) to support the view that the osteoclast originates from a hematopoietic progenitor. Nevertheless the precise identity of the osteoclast progenitor and its relationship to the monocyte/macrophage lineage are not fully understood.

Considerable information on the interactions between stromal tissue and hematopoietic cells in both marrow and spleen has been obtained from tissue culture systems. In long-term bone marrow cultures (LTBMCs), the stromal monolayer includes reticular cells, endothelial cells, preosteoblasts, and adipocytes, which form a microenvironment to support proliferation and differentiation of hematopoietic cells (6, 7).

The purpose of these studies was to determine whether such cells of the marrow stromal microenvironment play a role in the regulation of osteoclastogenesis. Hematopoietic progenitors were cocultured with a cloned normal stromal cell line (GBLneo^r) as a

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representative cell type of the marrow microenvironment that supports hematopoiesis *in vitro*. We have shown that hematopoietic progenitors free from stromal cells differentiate to osteoclast-like multinucleated cells in response to M-CSF and GM-CSF or IL-3 (8). Their differentiation is further enhanced by the bone matrix protein osteocalcin (9). In this study, we demonstrate that PTH and 1,25-dihydroxyvitamin D_3 have indirect effects on osteoclastogenesis, mediated through the stromal compartment. Furthermore, a stromal cell line from osteopetrotic (*op/op*) mice failed to mediate these indirect effects on osteoclast differentiation.

Materials and Methods

Long-Term Bone Marrow Cultures. Long-term bone marrow cultures (LTBMCs) were established from six-week-old C57B16/J male mice (Jackson Laboratories, Bar Harbor, ME) according to the Greenberger modification (10, 11) of Dexter's method (12). The medium consisted of Fischer's leukemic medium (GIBCO, Grand Island, NY), 100 U/ml penicillin (GIBCO), 100 μg/ml streptomycin (GIBCO), 10^{-5} *M* hydrocortisone sodium succinate (Abbott Laboratories, North Chicago, IL), and 20% horse serum (Hazelton, St. Lenexa, KS). Complete medium changes were made at weekly intervals, and, after four weeks, horse serum was replaced by 20% heat-inactivated fetal bovine serum (FBS; GIBCO, Grand Island, NY). Heat-inactivation was accomplished by heating the serum to 56°C for 45 min. The flasks were incubated in a humid atmosphere at 33°C and in 5% CO_2 in air. After five weeks, the nonadherent (NA) cells were collected at weekly intervals.

Characterization of the Nonadherent Cell Population Derived From Long-Term Bone Marrow Cultures. The nonadherent cells harvested from long-term bone marrow cultures were washed and counted to determine the viability by the Trypan blue dye exclusion method. Smears of the NA cells were made in a cytospin centrifuge (Shandon Southern Products Ltd., Cheshire, England). The cytospins were air dried and fixed with 100% methanol and stained with Wright's modified Giemsa stain (Sigma Diagnostics WH-16, St. Louis, MO). Differential counts were obtained by counting 200–300 cells.

GM-CFUc Assay. Suspensions of cells were made in α-MEM (Minimum Essential Medium Alpha; GIBCO) with 0.8% methyl cellulose, 10% fetal calf serum, 10^{-2} *M* β-mercaptoethanol (Terry Fox Laboratories, Vancouver, BC) or GIBCO BRL Stem Cell Kit with 1 ng/ml recombinant mouse granulocyte macrophage–colony stimulating factor (rm GM-CSF; Genzyme, Boston, MA). The cells were seeded at 0.5 and at 1×10^5 cells/ml in 35 mm tissue culture dishes. The cultures were incubated in humidity chambers at 37°C and in 5% CO_2 in air for seven days. Colonies of >40 cells were counted with a phase-contrast, inverted microscope at ×4 magnification (Nikon Inc., Garden City, NY). This assay was also used to count colonies generated by whole bone marrow and fractionated bone marrow.

Colorimetric Assay for Cell Number. Cellular proliferation was assayed by Mosman's technique (13). Cells were plated into 96-well plates (flat-bottomed, Falcon®, NJ) at 2×10^5 viable cells/well. After the treatment periods, media were removed and 100 μl of Dulbecco's Modified Eagle Medium (DMEM; GIBCO), 10% FBS, and 10% MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrasolium bromide; Chemicon, Temecula, CA) was added to each well. The wells were incubated for 3–4 hr at 37°C and in 5% CO_2 in air. One hundred microliters of the color development solution (isopropanol with 0.04 *N* HCl) was added, and the contents of each well were thoroughly mixed by repeat pipetting. The absorbance of each well was measured at 600 nm on a plate reader (EL308; Bio-Tek Instruments Inc., Winooski, VT).

Differentiation-Inducing Cell Culture Experiments. Cultures were established with whole bone marrow (WBM) from C57B16/J mice by seeding a monocellular suspension of marrow cells at 4×10^5 cells/0.8 cm^2 in 8-chamber slides (Permanox®, Lab-Tek®, Naperville, IL) in basal α medium. This consisted of α-MEM, 2% heat-inactivated horse serum (HS; Hazleton or GIBCO), 100 U/ml penicillin (GIBCO), and 100 μg/ml streptomycin (GIBCO). After a 24-hr preincubation period, basal medium was replaced by treatment medium. The treatment medium consisted of basal α medium supplemented with 2% or 5% heat-inactivated horse serum and growth factors including 10% L-cell-conditioned medium (LC-CM) as a source of murine M-CSF. LC-CM was prepared by collecting supernatant from cultures of confluent L-929 cells grown in RPMI medium (14), centrifugation at 1600 rpm for 10 min, and storage at −80°C. The following factors were used in the treatment media: recombinant mouse granulocyte macrophage–colony stimulating factor (GM-CSF; Genzyme, Boston, MA and Collaborative Biomedicals, Medford, MA), recombinant human macrophage–colony stimulating factor (M-CSF; GIBCO BRL, Gaithersburg, MD), 1,25-dihydroxyvitamin D_3 (Hoffman, La Roche, NY), and bovine 1-34 Parathyroid hormone (PTH; Peninsula Labs. Inc., Belmont, CA). Cultures received half-media changes twice each week. Cultures were evaluated after two weeks treatment.

Similar cultures were established with nonadherent cells collected from LTBMCs. These hematopoietic cells were designated as including putative pro-

genitors (PPs) of osteoclasts and treated as above. The half-media changes prevented loss of cells which became adherent with time.

Cocultures of Progenitors with Stromal Cell Lines. Nonadherent cells from LTBMCs were cocultured with plateau phase monolayers of the GBLneo^r stromal cell line to determine the effect of stromal cells on osteoclastogenesis. A permanent cloned stromal cell line (GBL/6) and its sub-clone GBLneo^r containing the neo^r gene were selected and expanded *in vitro* as described (15). A marrow stromal cell line was established from an *op/op* osteopetrotic mouse (from the mouse colony of Dr. Sandy Marks), according to the methods described by Anklesaria *et al.* (16, 17). The cells were maintained in DMEM, 10% FBS, 100 U/ml penicillin (GIBCO), 100 μg/ml streptomycin (GIBCO), and 10^{-5} *M* hydrocortisone sodium succinate (Abbott Laboratories) at 33°C in 5% CO_2 in air. The stromal cell lines were cultured in eight-chamber slides and 24-well plates for mixed culture experiments. When they reached confluence, the medium was gently removed and a suspension of putative osteoclast progenitors in α-MEM medium was seeded on this confluent stromal layer at a density of 4×10^5 cells/0.8 cm^2. After 24 hr at 37°C and in 5% CO_2 in air, the basal medium was replaced by treatment medium and the cultures maintained for two weeks with biweekly, half-medium changes. In some experiments, L-cell conditioned medium was omitted from treatment medium, but under these circumstances, serum was increased to 5% heat-inactivated horse serum to ensure cellular viability.

Multinuclearity and Tartrate-Sensitive and Tartrate-Resistant Acid Phosphatase Activity. After two weeks of treatment, cultures were rinsed with phosphate-buffered saline (PBS; GIBCO) and fixed for 1 min with cold acetone:methanol (1:1). The wells were rinsed thoroughly with distilled water following which 400 μl of fresh filtered incubation medium was added to the wells for 60 min at 37°C. The incubation medium consisted of 25 ml each of distilled water and 0.2 *M* acetate buffer (pH 5.2), 5 mg naphthol AS-BI phosphate (Sigma) in 0.2 ml dimethylformamide (Sigma), and 60 mg fast red violet LB salt (Sigma). For assessment of acid phosphatase (AP) and tartrate-resistant acid phosphatase (TRAP), respectively, either zero or 20 m*M* tartrate (Sigma) was included in the incubation medium followed by two drops of 10% manganese chloride (Sigma). The incubation medium without tartrate was added to half the number of wells. The stained cells in these wells represented acid phosphatase (AP)–positive cells. The stained cells in the wells that received the incubation medium with tartrate represented TRAP-positive cells. After incubation, the wells were washed thoroughly with water and coverslipped with Aquapolymount® (Polysciences, Inc., Warrington, PA). Positive cells exhibited pink cytoplasm. Quantitation was accomplished by counting the number of stained and unstained multinucleated cells using a light microscope (Nikon). Multinucleation was assessed by counting the cells with three or more nuclei. Cells were counted at ×20 magnification in 16 contiguous fields along a diagonal pathway in each well—a method devised to account for the non-uniform distribution of cells within wells, especially seen in basal groups (8). The TRAP-stained multinucleated cells represented osteoclast-like cells and the tartrate-sensitive multinucleated cells (i.e., unstained in the presence of tartrate) represented macrophage polykaryons. The technique was verified by comparison with freshly isolated mouse calvarial osteoclasts, prepared from neonatal mice by a procedure modified from Chambers and Magnus (18). Statistical significance was tested by nonparametric (Kruskal-Wallis modification) analysis of variance with Statview (TM) software.

TRAP activity was quantified by a microphotometric technique using a Nikon Phoscan P1 photometer that was coupled to a Nikon Labphot light microscope and a Compac® computer (8). Transmitted light was measured through 40–50 contiguous fields (0.64 mm^2 each) at ×4 magnification and expressed as absorbance per mm^2. For each culture, the average of all fields and the average for the most intensely stained 30th percentile were calculated. The 30th percentile data is a more robust statistic for describing skew distributions (8). Analysis of variance (ANOVA) was used to analyze the data.

Results

Osteoclastogenesis in Short-Term Whole Bone Marrow Cultures. In whole bone marrow (WBM) that was cultured for two weeks in the presence of LC-CM as a source of soluble M-CSF, parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3, or GM-CSF promoted both multinucleation and the formation of TRAP-positive multinucleated cells (Table I, Fig. 1). There was a 1.2-fold increase in the number of multinucleated cells in cultures with PTH or 1,25-dihydroxyvitamin D_3 compared with the control group ($P < 0.05$), while GM-CSF increased multinucleation by 1.7-fold ($P < 0.05$). A greater number of multinucleated cells was generated in cultures with a combination of GM-CSF and PTH (Table I, $P < 0.05$).

These agents had more dramatic effects upon the differentiation of osteoclast-like multinucleated cells than on multinuclearity. PTH increased the number of TRAP-positive multinucleated cells by 3.9-fold, GM-CSF also increased the number of TRAP-positive multinucleated cells by 3.9-fold, and 1,25-dihydroxy-

Table I. Effect of GM-CSF ± PTH or 1,25-Dihydroxyvitamin D_3 on Multinuclearity and the Number of TRAP-Positive Multinucleated Cells in Whole Bone Marrow Cultures

	L10			L10 + GM-CSF		
	0	PTH	1,25 D_3	0	PTH	1,25 D_3
# Cells	81.7 ± 5.7	80.1 ± 7.7	100.5 ± 2.1	70.1 ± 2.3	84.5 ± 1.4	90.6 ± 2.6
# MNCs	9.5 ± 0.6	11.7 ± 0.1	18.9 ± 0.1*	13.3 ± 0.7	18.9 ± 1.9*	16.4 ± 0.9*
# $TRAP^{+ve}$ MNCs	2.3 ± 0.4	8.8 ± 0.4*	8.8 ± 0.4*	7.7 ± 0.5*	14.7 ± 1.1*†	12.3 ± 0.4*†
% MNCs/total cells	11.7 ± 0.2	14.5 ± 1.6*	14.3 ± 0.7*	19.6 ± 1.4*	22.3 ± 0.0*	17.6 ± 0.2*
% $TRAP^{+ve}$/total cells	2.8 ± 0.4	11.0 ± 1.1*	8.7 ± 0.2*	11.0 ± 0.4*	17.4 ± 0.0*†	13.1 ± 0.1*
% $TRAP^{+ve}$/total MNCs	24.0 ± 0.3	75.9 ± 3.4*	60.9 ± 0.6*	55.9 ± 0.1*	77.8 ± 3.4*	77.4 ± 2.1*

Basal L10 medium contained α-MEM with 2% heat-inactivated horse serum and 10% L-cell-conditioned medium. PTH was used at 10^{-8} *M;* 1,25-dihydroxyvitamin D_3 was used at 10^{-8} *M;* GM-CSF was used at 5 U/ml. Total cells is the number of cells per field, calculated as the average of 16 fields, and expressed as the mean ± SD of four wells. MNCs refers to the number of multinucleated cells with three or more nuclei and is expressed as the average number of cells per field and as % of total cell number. $TRAP^{+ve}$ refers to tartrate-resistant acid phosphatase–positive multinucleated cells and is expressed as the number per field and as the mean % of total cell number and as % of the number of multinucletaed cells per field.
* $P < 0.05$ vs L10.
† $P < 0.05$ vs GM.

vitamin D_3 increased the number of TRAP-positive multinucleated cells by 3.1-fold, compared with the basal L10 group ($P < 0.05$). Cultures with GM-CSF and PTH generated the greatest number of multinucleated cells and TRAP-positive multinucleated cells (Table I). The percent of multinucleated cells that demonstrated tartrate-resistance was high in all treatment groups, between 56% and 78%; the low percent in basal L10 medium indicates that those multinucleated cells have the phenotype of tartrate-sensitive macrophage polykaryons. Other studies (8, 9) demonstrated that TRAP-positive multinucleated cells displayed other features of the osteoclast phenotype including ruffled borders and responsiveness to calcitonin. These results indicate that GM-CSF, PTH or 1,25-dihydroxyvitamin D_3 can promote osteoclastogenesis in short-term whole bone marrow cultures.

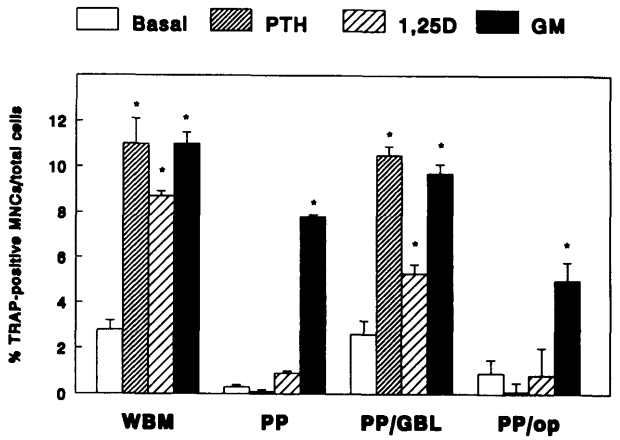

Figure 1. Comparison of osteoclastogenic effects of PTH (10^{-8} *M*), 1,25-dihydroxyvitamin-D_3 (10^{-8} *M*), and GM-CSF (5 U/ml) in cultured whole bone marrow (WBM), putative osteoclast progenitors derived from long-term bone marrow cultures (PP), cocultures of PPs with normal stromal cell line (PP/GBL), and cocultures of PPs with osteopetrotic stroma (PP/*op*). The number of tartrate-resistant acid phosphatase–positive multinucleated cells was assessed after two weeks and is expressed as % of total cell number ± SD with $n = 4$. *Significantly different from basal medium which contains 10% LC-CM as a source of M-CSF, $P < 0.05$.

Osteoclastic Differentiation of Hematopoietic Progenitors in the Absence of Stromal Cells. For differentiation studies, nonadherent cells were collected from LTBMCs between five to 12 weeks *in vitro*. This range provided consistent populations of an average of 6.8 million cells/flask/week. These nonadherent cells were composed of 70.8% ± 4.9% granulocytes, 19.7% ± 4.9% monocytes, and 9.6% ± 2.8% blasts. The critical feature of this population of hematopoietic cells is that it is free of stromal cells (8, 11, 14). Further, the long-term bone marrow culture-derived progenitors were enriched for CFU-GM-forming progenitors (0.33% ± 0.09%), compared with fresh whole bone marrow (0.15% ± 0.08%) and (Ficoll-hypaque) fractionated bone marrow (0.20% ± 0.03%). The majority of granulocytes did not survive in differentiation cultures for longer than two days.

In the presence of L10, GM-CSF promoted the development of multinucleated cells ($P < 0.05$) (Table II). In three separate experiments, GM-CSF increased multinuclearity up to 5.1-fold. 1,25-dihydroxyvitamin D_3 treatment was associated with smaller increases in multinuclearity (Table II). In one experiment, the number of tartrate-sensitive multinucleated cells (a measure of macrophage polykaryons) generated in the presence of 1,25-dihydroxyvitamin D_3 was 1.4-fold greater than control (Fig. 2A, PP group), but the number of TRAP-positive multinucleated cells (an index of the osteoclast-like cells) did not increase significantly in the presence of 1,25-dihydroxyvitamin D_3 (Fig. 1; Fig. 2B, PP group). In cultures of progenitors alone, 1,25-dihydroxyvitamin D_3 and GM-CSF each increased multinucleation, but only GM-CSF promoted

Table II. Effect of GM-CSF ± PTH or 1,25-Dihydroxyvitamin D_3 on Multinuclearity and the Number of TRAP-Positive Multinucleated Cells in Putative Progenitors Derived from Long-Term Bone Marrow Cultures

	L10			L10 + GM-CSF		
	0	PTH	1,25 D_3	0	PTH	1,25 D_3
# Cells	96.5 ± 0.7	91.2 ± 0.9	99.7 ± 3.8	92.7 ± 1.6	92.4 ± 1.2	93.1 ± 2.1
# $TRAP^{+ve}$ MNCs	0.3 ± 0.1	0.1 ± 0.1	0.9 ± 0.1	7.2 ± 0.3*	6.9 ± 0.7*	5.4 ± 0.1*
% MNCs/total cells	2.6 ± 0.1	0.6 ± 0.1	4.0 ± 0.8*	13.3 ± 1.6*	12.2 ± 0.9*	9.6 ± 0.5*
% $TRAP^{+ve}$/total cells	0.3 ± 0.1	0.1 ± 0.2	0.9 ± 0.1	7.8 ± 0.8*	7.5 ± 0.6*	5.6 ± 0.9*
% $TRAP^{+ve}$/total MNCs	2.2 ± 0.3	15.4 ± 1.3*	23.3 ± 1.7*	56.5 ± 2.1*	61.3 ± 2.3*	60.5 ± 1.8*

Basal L10 medium contained α-MEM with 2% heat-inactivated horse serum and 10% L-cell-conditioned medium. PTH was used at 10^{-8} *M;* 1,25-dihydroxyvitamin D_3 was used at 10^{-8} *M;* GM-CSF was used at 5 U/ml. Total cells is the number of cells per field, calculated as the average of 16 fields, and expressed as the mean ± SD of four wells. MNCs refers to the number of multinucleated cells with three or more nuclei and is expressed as % of total cell number. $TRAP^{+ve}$ refers to tartrate-resistant acid phosphatase–positive multinucleated cells and is expressed as the number per field and as the mean % of total cell number and as % of the number of multinucleated cells per field.
* $P < 0.05$ vs L10.

the formation of TRAP-positive multinucleated cells, as high as 26-fold. Although a combination of GM-CSF and PTH or GM-CSF and 1,25-dihydroxyvitamin D_3 increased the number of TRAP-positive multinucleated cells, this increase was equivalent to the direct effect of GM-CSF alone on the putative progenitors. Similar results were observed in two other experiments. In one experiment, TRAP activity was quantified by the microphotometric technique and was found to be doubled in the presence of GM-CSF ($P < 0.05$, Fig. 3).

Thus, in contrast to short-term whole bone marrow cultures, osteoclastic differentiation of hematopoietic progenitors derived from LTBMCs was not stimulated by PTH nor 1,25-dihydroxyvitamin D_3. GM-CSF, however, had direct effects on differentiation of the progenitors.

Osteoclastogenesis in Cocultures of Putative Progenitors From LTBMCs with a Cloned Stromal Cell Line (GBLneo^r). Hematopoietic progenitors were cocultured with the GBLneo^r stromal cell line, which is known to support hematopoietic stem cells (15). In three separate experiments, PTH significantly increased multinuclearity up to 1.6-fold. GM-CSF also increased multinuclearity up to 1.6-fold (Table III). Unlike the experiments in which the progenitors were cultured without stromal cells, PTH and 1,25-

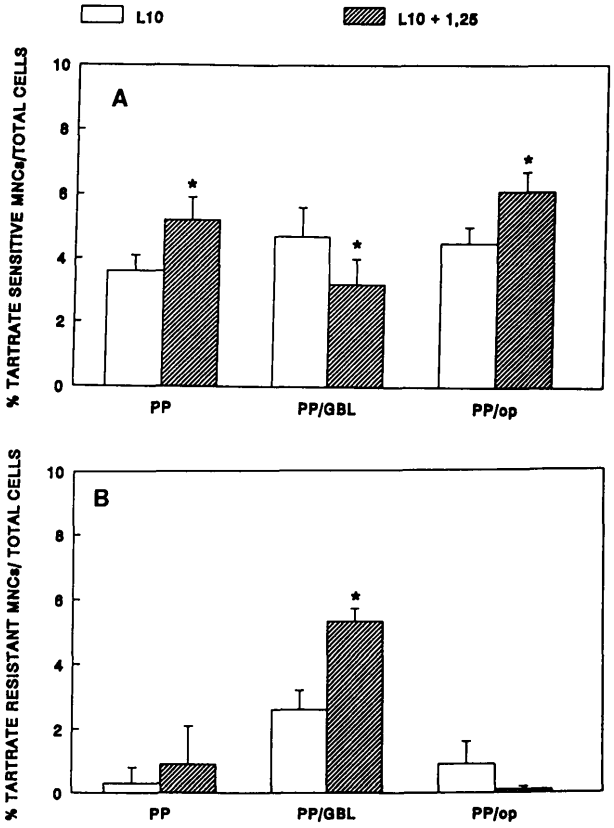

Figure 2. Comparison of effects of 10^{-8} *M* 1,25-dihydroxyvitamin D_3 on generation of (A) tartrate-sensitive and (B) tartrate-resistant acid phosphatase–positive multinucleated cells in cultured putative osteoclast progenitors (PP), cocultures of PPs with normal stromal cell line (PP/GBL), and cocultures of PPs with osteopetrotic stroma (PP/*op*) after two weeks. *Significantly different from basal L10, $P < 0.05$.

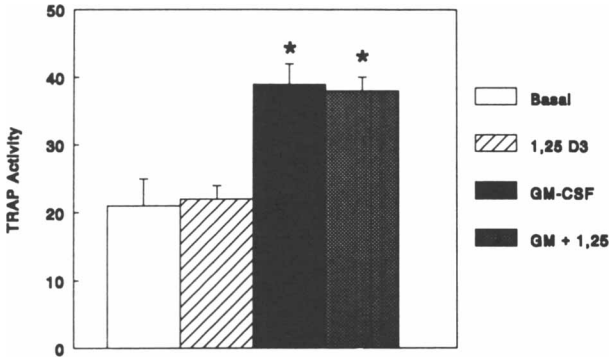

Figure 3. Measurement of tartrate-resistant acid phosphatase (TRAP) activity. Activity was quantified by microphotometry in cultures of progenitors treated for two weeks with 5 U/ml GM-CSF and/or 10^{-8} *M* 1,25-dihydroxyvitamin D_3. Activity is expressed as absorbance × $10^3/mm^2$ for the mean ± SD of 4 replicates. Basal is L10 medium, 1,25 D_3 is 1,25-dihydroxyvitamin D3 at 10^{-8} *M*, and GM-CSF is at 5 U/ml.

Table III. Effect of GM-CSF, PTH, or 1,25-Dihydroxyvitamin D_3 on Multinuclearity and the Number of TRAP-Positive Multinucleated Cells in Cocultures of Putative Progenitors with GBLneo^r Stromal Cells

	L10			L10 + GM-CSF		
	0	PTH	1,25 D_3	0	PTH	1,25 D_3
# Cells	92.3 ± 0.8	85.6 ± 0.3	71.4 ± 0.7	81.6 ± 0.6	90.3 ± 1.7	99.4 ± 1.1
# MNCs	6.7 ± 0.9	10.1 ± 0.5	6.3 ± 0.7	10.0 ± 3.9	10.1 ± 0.5	11.2 ± 0.4*
# TRAP^{+ve} MNCs	2.4 ± 0.5	9.0 ± 0.3*	3.9 ± 0.3	7.9 ± 0.3*	9.9 ± 0.3*	9.6 ± 0.4*
% MNCs/total cells	7.2 ± 0.6	11.8 ± 0.5*	8.6 ± 0.9	12.0 ± 0.7*	12.0 ± 0.6*	11.3 ± 0.2
% TRAP^{+ve}/total cells	2.6 ± 0.6	10.5 ± 0.3*	5.3 ± 0.4*	9.7 ± 0.4*	11.1 ± 0.5*	9.7 ± 0.4*
% TRAP^{+ve}/total MNCs	35.9 ± 0.7	89.3 ± 1.2*	62.0 ± 2.0*	80.9 ± 0.8*	92.0 ± 1.0*	85.5 ± 0.6*

Basal L10 medium contained α-MEM with 2% heat-inactivated horse serum and 10% L-cell-conditioned medium. PTH was used at 10^{-8} *M;* 1,25-dihydroxyvitamin D_3 was used at 10^{-8} *M;* GM-CSF was used at 5 U/ml. Total cells is the number of cells per field, calculated as the average of 16 fields, and expressed as the mean ± SD of four wells. MNCs refers to the number of multinucleated cells with three or more nuclei and is expressed as the average number of cells per field and as % of total cell number. TRAP^{+ve} refers to tartrate-resistant acid phosphatase–positive multinucleated cells and is expressed as the number per field and as the mean % of total cell number and as % of the number of multinucleated cells per field.
* $P < 0.05$ vs L10.

dihydroxyvitamin D_3 promoted the formation of TRAP-positive multinucleated cells in cocultures (Fig. 1, Fig. 2B). This stimulation occurred with a reduction in the number of tartrate-sensitive macrophage polykaryons (Fig. 2A; PP/GBL group). In these cocultures, GM-CSF promoted the formation of TRAP-positive multinucleated cells up to 3.7-fold, and also displayed an additive effect in combination with PTH up to 4.2-fold ($P < 0.05$, Table III). PTH promoted the formation of TRAP-positive multinucleated cells up to 4-fold and 1,25-dihydroxyvitamin D_3 promoted the formation of TRAP-positive multinucleated cells up to 2-fold. These results indicate that while GM-CSF had a direct effect, PTH and 1,25-dihydroxyvitamin D_3 acted in the presence of the GBLneo^r cell line to promote osteoclastogenesis. Thus, the GBLneo^r cell line supported the actions of these agents on osteoclastogenesis as had been seen with short-term whole bone marrow cultures (Fig. 1).

All of the above studies were done in the presence of 10% LC-CM to ensure viability of the progenitors. We considered the possibility that contact of progenitors with stromal cells substitutes for exogenous M-CSF provided in LC-CM. As a test of this hypothesis, progenitors were cocultured with GBLneo^r in the absence of LC-CM. To ensure viability of cells under these conditions, we increased the concentration of horse serum to 5%. In these cocultures of progenitors with GBLneo^r stroma, GM-CSF promoted multinucleation by 1.9-fold and the formation of TRAP-positive multinucleated cells by 4.2-fold (Fig. 4). PTH or 1,25-dihydroxyvitamin D_3 also promoted multinuclearity by 1.8-fold and 1.2-fold respectively. PTH increased the formation of TRAP-positive multinucleated cells by 4.5-fold and 1,25-dihydroxyvitamin D_3 increased the formation of TRAP-positive multinucleated cells by 1.9-fold. Thus, progenitors, which had been shown to require M-CSF for proliferation and differentiation (8), did not have this requirement when they were cocultured with marrow stromal cells.

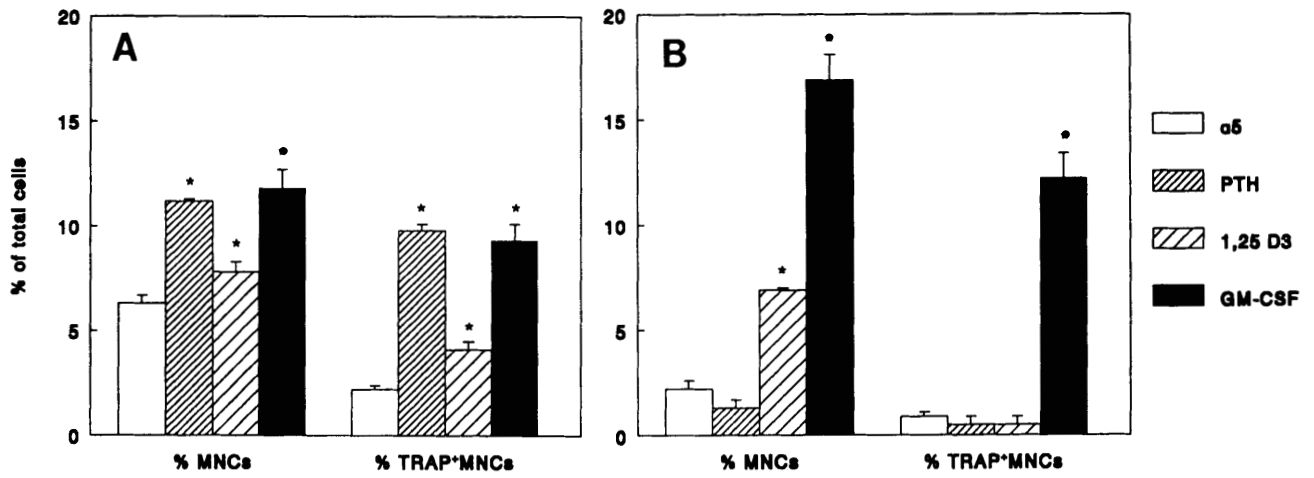

Figure 4. Effects of 10^{-8} *M* PTH, 10^{-8} *M* 1,25-dihydroxyvitamin D_3 or 5 U/ml GM-CSF on multinuclearity (MNCs) and % TRAP-positive multinucleated cells in the absence of soluble M-CSF. Progenitors were cocultured in the absence of LC-CM for two weeks with (A) GBLneo^r stroma or (B) *op/op* stroma. *Significantly different from basal α medium supplemented with 5% horse serum (α5), $P < 0.05$.

Altered Osteoclastogenesis in Cocultures of Progenitors with a Stromal Cell Line from an Osteopetrotic (*op/op*) Mouse. To directly determine the importance of stromal M-CSF in differentiation of progenitors, we generated marrow stromal cells from osteopetrotic (*op/op*) mice. The *op/op* mutant is characterized by the absence of functional M-CSF and thus enabled us to test the hypothesis that this factor was essential for the actions of PTH or 1,25-dihydroxyvitamin D on cocultures. Experiments were carried out with normal progenitors cocultured with *op/op* stromal cells in the presence of soluble M-CSF alone (L10). PTH did not enhance multinuclearity nor osteoclast differentiation of progenitors (Table IV, Fig. 1). 1,25-Dihydroxyvitamin D_3 promoted the formation of TRAP-sensitive multinucleated cells to a small extent (35%) but did not promote the formation of TRAP-positive multinucleated cells (Fig. 2A, B, PP/*op* group). GM-CSF increased both multinuclearity and the formation of TRAP-positive multinucleated cells. The stimulatory effect of GM-CSF on multinuclearity was by almost 1.5-fold and that on TRAP-positive multinucleated cells was 5.5-fold (Table IV). Thus, the pattern of responsiveness of the progenitors on *op/op* stromal cells was similar to progenitors alone (Fig. 1).

A set of experiments was carried out in the absence of LC-CM to allow comparison with cocultures of progenitors with the normal GBLneo stromal cell line, also under the conditions without LC-CM (Fig. 4). In cultures with *op/op* stroma, GM-CSF promoted multinuclearity by 7.7-fold and the formation of TRAP-positive multinucleated cells by 13.5-fold. This result was consistent with the direct effect of GM-CSF on progenitors cultured alone. PTH in these cocultures had no significant effect on either multinuclearity or the number of TRAP-positive multinucleated cells. In one experiment, 1,25-dihydroxyvitamin D_3 promoted multinuclearity 3-fold (Fig. 4B) with nearly all the multinucleated cells being *sensitive* to tartrate; these data indicate that in these cocultures with mutant stroma, 1,25-dihydroxyvitamin D_3 favored the formation of macrophage polykaryons.

Thus, under both conditions, with and without exogenous LC-CM, stromal cells derived from osteopetrotic *op/op* mouse marrow did not support the actions of PTH or 1,25 dihydroxyvitamin D_3 that were detected in short-term whole bone marrow or cultures with the GBL neo^r cell line. Further, the *op/op* stroma cell line did not enhance the direct effects of GM-CSF on multinuclearity and on differentiation of TRAP-positive multinucleated cells.

Discussion

A variety of culture systems have been used to examine the regulation of osteoclastic differentiation. Osteoclast formation in liquid cultures of bone marrow cells has been shown to be stimulated by 1,25-dihydroxyvitamin D_3 (19–26). However, such cultures were comprised of a heterogeneous population of stem cells with stromal cells. We chose to use the murine long-term bone marrow culture system as a source for these progenitors. The long-term bone marrow culture system is advantageous because it generates a large and constant population of viable progenitor cells enriched in CFU-GMs that are stroma-free. Thus, it was possible to study the direct effects of growth factors and cytokines on nonadherent progenitors alone or in contact with cloned stromal cell lines. Although several investigators (27, 28) used multinucleation as an index of osteoclastogenesis in marrow cultures, the fact that macrophage polykaryons are also multinucleated complicates interpretation (29, 30). We show that a subpopulation of multinucleated cells generated from nonadherent progenitors may be identified as macrophage polykaryons based upon tartrate-sensitivity. Previously, we showed that presumptive TRAP-positive multinucleated osteoclasts display ruffled borders and clear zones when cultured with bone (8) and

Table IV. Effects of GM-CSF, PTH, or 1,25-Dihydroxyvitamin D_3 on Multinuclearity and the Number of TRAP-Positive Multinucleated Cells in Cocultures of Putative Progenitors and *op/op* Stomal Cells

	L10			L10 + GM-CSF		
	0	PTH	1,25 D_3	0	PTH	1,25 D_3
# Cells	86.9 ± 2.3	88.3 ± 2.2	88.6 ± 1.6	87.2 ± 1.8	88.7 ± 0.6	90.5 ± 0.4
# MNCs	4.7 ± 0.2	4.5 ± 0.4	5.5 ± 0.5	7.9 ± 0.8	8.2 ± 0.6*	5.2 ± 0.8
# TRAP^{+ve} MNCs	0.8 ± 0.6	0.1 ± 0.2	0.1 ± 0.1	4.4 ± 0.8*	4.1 ± 0.5*	0.0 ± 0.0
% MNCs/total cells	5.4 ± 0.3	5.1 ± 0.4	6.2 ± 0.6	8.9 ± 0.8*	9.2 ± 0.6*	6.6 ± 0.6*
% TRAP^{+ve}/total cells	0.9 ± 0.2	0.1 ± 0.1	0.0 ± 0.0	5.0 ± 0.3*	4.6 ± 0.5*	3.6 ± 0.1*
% TRAP^{+ve}/total MNCs	16.9 ± 1.3	2.2 ± 0.3*	0.0 ± 0.0*	55.6 ± 2.3*	50.0 ± 1.7*	54.5 ± 2.1*

Basal L10 medium contained α-MEM with 2% heat-inactivated horse serum and 10% L-cell-conditioned medium. PTH was used at 10^{-8} *M;* 1,25-dihydroxyvitamin D_3 was used at 10^{-8} *M;* GM-CSF was used at 5 U/ml. Total cells is the number of cells per field, calculated as the average of 16 fields, and expressed as th emean ± SD of four wells. MNCs refers to the number of multinucleated cells with three or more nuclei and is expressed as the average number of cells per field and as % of total cell number. TRAP^{+ve} refers to tartrate-resistant acid phosphatase–positive multinucleated cells and is expressed as the number per field and as the mean % of total cell number and as % of the number of multinucleated cells per field.
* $P < 0.05$ vs L10.

respond to calcitonin (9). Although there is no single definitive marker of the osteoclastic phenotype *in vitro,* the development of TRAP-positive multinucleated cells has been commonly used as a quantitative screen for detecting differentiation in cultured hematopoietic cells. It is possible that some of these markers are an indication of the early steps in osteoclastogenesis, later stages requiring contact with bone matrix components or other regulatory agents (9).

Our observations with short-term whole bone marrow cultures were similar to those of other investigators (19–25). With whole bone marrow, all factors including GM-CSF, PTH, and 1,25-dihydroxyvitamin D_3 promoted the formation of osteoclastic cells. In such heterogeneous cultures, differentiation is regulated indirectly by adherent cells, including those of the osteoblastic lineage (19–31). Other investigations comparing spleen and marrow cultures indicated that a contract-dependent interaction between the stroma and the stem cells is necessary for osteoclastogenesis (32).

These culture conditions were designed to control for proliferation of progenitors under the different treatment conditions so that differentiation effects would be independent of cell density (8). In cocultures of hematopoietic progenitor cells with the cloned GBLneo^r stromal cell line, 1,25-dihydroxyvitamin D_3 promoted the formation of osteoclast-like cells, an effect similar to that seen in whole bone marrow cultures. Similarly, PTH promoted the formation of TRAP-positive multinucleated cells in whole bone marrow cultures and in cocultures of progenitors with the GBLneo^r stroma, but not from progenitors cultured alone. All observations taken together indicate that in the presence of M-CSF, GM-CSF has a direct action on progenitors to promote osteoclastogenesis (8). These new data show that 1,25-dihydroxyvitamin D_3 and PTH have an indirect (stromal-mediated) action on osteoclastogenesis. We conclude that this coculture system with normal GBLneo^r stromal and a population of hematopoietic progenitors obtained free of stromal elements from long-term bone marrow cultures serves as a partially defined model for whole bone marrow.

Osteopetrosis is an heredity disorder characterized by normal appositional bone growth, but failure of physiologic bone resorption. In the *op/op* osteopetrotic strain of mice, the mutation was suggested to be a defect in local microenvironment in bone because the disease cannot be cured by bone marrow transplantation (33, 34). Wiktor-Jedrzejczak *et al.* proposed that this defect in the *op/op* mutant mouse may be due to the failure of hematopoietic stromal cells to supply macrophage–colony stimulating factor (M-CSF) (35). Felix et al. showed that cultured osteoblasts and fibroblasts from this mutant do not shown M-CSF activity and that resident macrophages are absent in bone marrow (36). Yoshida *et al.* reported that the deficiency in M-CSF production is due to a mutation in the M-CSF gene in which a stop codon is inserted early in the coding region (37). Osteoclast formation was shown to be induced in *op/op* mice by administration of pharmacological doses of M-CSF (36). On the other hand, Wiktor-Jedrzejczak *et al.* found that intraperitoneal implantation of L-cells in diffusion chambers induced many peritoneal macrophages and restored the circulating levels of M-CSF to near normal levels, but did not cure osteopetrosis (35). He speculated that contact between the M-CSF producing stromal cell and the osteoclast progenitor may be required to correct that part of the defect. We found that when normal mouse osteoclast progenitors were cocultured with the osteopetrotic (*op/op*) stromal cells, the indirect or stroma-mediated effects of 1,25-dihydroxyvitamin D_3 and PTH were conspicuously missing. Thus, the *op/op* marrow stromal cell line lacked the ability to support osteoclastogenesis. Supplementation with L-cell-conditioned medium as a source of soluble M-CSF did not correct this deficiency (Fig. 1, Table IV). Using qualitative indices of osteoclastogenesis, Kodama *et al.* reported that 1,25-dihydroxyvitamin D_3 failed to stimulate development of TRAP-positive multinucleated cells in cocultures of whole bone marrow cells with a cloned cell line OP6L7 that was derived from calvarial bones from *op/op* mice (38). All of these findings suggest that close contact may be essential for osteoclastogenesis. This conclusion is similar to that of Stein *et al.*, who reported that the membrane-bound M-CSF expressed in NIH-3T3 fibroblasts effectively stimulated the growth of macrophages and concluded that membrane-associated M-CSF may be more effective than the exogenously added M-CSF in providing a suitable microenvironment for differentiation (39). The importance of cell-to-cell and cell-matrix contacts has been well established for the hematopoietic microenvironment (10, 12, 40–43). The close association of hematopoietic progenitors with stromal cells may possibly allow the stem cells to respond to very low levels of secreted growth factors, or enable cell-to-cell transfer of mediators. Differentiation-inducing factors are known to be produced by primary stromal cells and may be effective in their matrix/membrane-bound form (44–47).

These results indicate that stimulation of osteoclastic differentiation by PTH or 1,25-dihydroxyvitamin D_3 is mediated through normal stromal cells. It is suggested that stromal-derived M-CSF may contribute to these indirect effects because of the finding that *op/op* stromal cells which are deficient in M-CSF do not support these regulatory mechanisms. This *in vitro* model of defined compartments of the marrow microenvironment may be useful in determining the mech-

anisms of actions of other differentiation-inducing factors on osteoclastogenesis.

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1. Kahn AJ, Simmons DJ. Investigation of cell lineage in bone using a chimera of chick and quail embryonic tissue. Nature **258**:325–327, 1975.
2. Fischman DA, Hay ED. Origin of osteoclasts from mononuclear leucocytes in regenerating newt limbs. Anat Rec **143**:329–339, 1962.
3. Jee WSS, Nolan PD. Origin of osteoclasts from the fusion of phagocytes. Nature (London) **200**:225–227, 1963.
4. Walker DG. Bone resorption restored in osteopetrotic mice by transplantation of normal bone marrow and spleen cells. Science **190**:784–789, 1975.
5. Coccia PF, Kririt W, Cervenka J, Clawson C, Kersey JH, Kim TH, Nesbit ME, Ramsay KC, Warkentin PL, Teitelbaum SL, Kahn AJ, Brown EM. Successful bone marrow transplantation for infantile malignant osteopetrosis. N Engl J Med **302**:701–708, 1980.
6. Sorrell JM, Weiss L. Cell interactions between hematopoietic and stromal cells in the embryonic chick bone marrow. Anat Rec **197**:1–19, 1980.
7. Owen M. The origin of bone cells in postnatal organism. Arthritis Rheum **23**:1073–1080, 1980.
8. Liggett WH, Shevde N, Anklesaria P, Sohoni S, Greenberger JS, Glowacki J. Effects of macrophage-colony stimulating factor and granulocyte-macrophage-colony stimulating factor on osteoclastic differentiation of hematopoietic progenitor cells. Stem Cells **11**:398–411, 1993.
9. Liggett WH Jr., Lian JB, Anklesaria P, Greenberger JS, Glowacki J. Effects of osteocalcin on proliferation and differentiation of putative osteoclast progenitors from murine long-term bone marrow cultures. J Bone Miner Res **5**:S78, 1990.
10. Greenberger JS. Sensitivity of corticosteroid-dependent, insulin-resistant, lipogenesis in marrow preadipocytes of obese-diabetic (db/db) mice. Nature **275**:752–754, 1978.
11. Greenberger JS. Long-term hematopoietic cultures. In: Golde DW, Ed. Hematopoiesis. New York: Churchill-Livingstone, pp 203–243, 1984.
12. Dexter TM, Allen TD, Lajtha LJ. Conditions controlling the proliferation of hemopoietic stem cells *in vitro*. J Cell Physiol **91**:335–344, 1977.
13. Mosman T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods **65**:55–63, 1983.
14. Sakakeeny MA, Greenberger JS. Granulopoiesis longevity in continuous bone marrow cultures and factor-dependent cell line generation: Significant variation among 28 inbred mouse strains and outbred stocks. J Natl Cancer Inst **68**:305–317, 1982.
15. Anklesaria P, Kase K, Glowacki J, Holland CA, Sakakeeny MD, Wright JA, Fitzgerald THJ, Lee C, Greenberger JS. Engraftment of a clonal bone marrow stromal cell line *in vivo* stimulates hematopoietic recovery from total body irradiation. Proc Natl Acad Sci USA **84**:7681–7685, 1987.
16. Anklesaria P, Klarsen V, Sakakeeny MA, Fitzgerald TJ, Harrison D, Rybak NE, Greenberger JS. Biological characterization of cloned permanent stromal cell lines from anemic S1/S1^d mice and +/+ littermates. Exp Hematol NY **15**:195–202, 1987.
17. Anklesaria P, Teixido J, Laiho M, Pierce JH, Greenberger JG. Cell-cell adhesion mediated by binding of membrane-anchored transforming growth factor to epidermal growth factor receptors promotes cell proliferation. Proc Natl Acad Sci **87**:3289–3293, 1990.
18. Chambers TJ, Magnus CJ. Calcitonin alters behaviour of isolated osteoclasts. J Pathol **136**:27–39, 1982.
19. Takahashi N, Yamana H, Yoshiki S, Roodman GD, Mundy GR, Jones SJ, Boyde A, Suda T. Osteoclast-like cell formation and its regulation by osteotropic hormones in mouse bone marrow cultures. Endocrinology **122**:1373–1382, 1988.
20. Kurihara N, Chenu C, Miller M, Civin C, Roodman GD. Identification of committed mononuclear precursors for osteoclast-like cells formed in long-term human marrow cultures. Endocrinology **126**:2733–2741, 1990.
21. Hattersley G, Chambers TJ. Generation of osteoclasts from hemopoietic cells and a multipotential cell line *in vitro*. J Cell Physiol **140**:478–481, 1989.
22. Hattersley G, Kerby JA, Chambers TJ. Identification of osteoclast precursors in multilineage hemopoietic colonies. Endocrinology **128**:259–262, 1991.
23. Roodman GD, Ibbotson KJ, MacDonald BR, Kuehl TJ, Mundy GR. 1,25(OH)$_2$-Vitamin D$_3$ causes formation of multinucleated cells with osteoclast characteristics in cultures of primate marrow. Proc Natl Acad Sci USA **82**:8213–8219, 1985.
24. Ibbotson KJ, Roodman GD, McManus CM, Mundy GR. Identification and characterization of osteoclast-like cells and their progenitors in cultures of feline marrow mononuclear cells. J Cell Biol **99**:471–480, 1984.
25. Sasaki T, Takahashi N, Higashi S, Suda T. Multinucleated cells from mouse bone marrow cells treated with 1,25-dihydroxyvitamin D$_3$ have ruffled borders and resorb dentin. Anat Rec **224**:379–387, 1989.
26. MacDonald BR, Takahashi N, McManus CM, Holahan J, Mundy GR, Roodman GD. Formation of multinucleated cells that respond to osteotropic hormones in long-term human bone marrow culture. Endocrinology **120**:2326–2333, 1987.
27. Addison WC. The distribution of nuclei in imprints of feline osteoclasts. J Anat **129**:63–70, 1979.
28. Reis WL, Gong KJ. A comparative study of osteoclasts: *In situ* versus smear specimens. Anat Rec **203**:221–226, 1982.
29. Hayashi T, Shinki T, Tanaka H, Abe E, Suda T. Polyamines are involved in 1,25-dihydroxyvitamin D$_3$ induced fusion of mouse alveolar macrophages. J Bone Miner Res **1**:235–241, 1986.
30. Kriepe H, Radzun HJ, Rudolph P, Barth J, Hansmann M, Heidorn K, Parwaresch MR. Multinucleated giant cells generated *in vitro*. Terminally differentiated macrophages with down regulated c-fms expression. Am J Path **130**:232–239, 1988.
31. Chambers TJ. The regulation of osteoclast development and function. In: Evered D, Harnett S, Eds. Cell and Molecular Biology of Vertebrate Hard Tissue, Symposium, No. 136. New York: Wiley, pp92–107, 1988.
32. Hattersley G, Chambers TJ. The role of bone marrow stroma in induction of osteoclastic differentiation. In: Cohn DV, Glorieux FH, Martin TJ, Eds. Calcium Regulation and Bone Metabolism. Elsevier Science Publishers, pp439–442, 1990.
33. Marks SC, Lane PW. Osteopetrosis, a new recessive skeletal mutation on chromosome 12 of the mouse. J Hered **67**:11–16, 1976.
34. Wiktor-Jedrzejczak W, Ahmed A, Szcylik C, Skelly RR. Hematological characterization of congenital osteopetrosis in op/op mouse. J Exp Med **156**:1516–1527, 1982.
35. Wiktor-Jedrzejczak W, Bartocci A, Ferrante AW Jr., Ahmed-Ansari A, Sell KW, Pollard JW, Stanley ER. Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. Proc Natl Acad Sci USA **87**:4828–4832, 1990.
36. Felix R, Cecchini MG, Fleisch H. Macrophage colony stimu-

lating factor restores *in vivo* bone resorption in the op/op osteopetrotic mouse. Endocrinology **127**:2592–2597, 1990.
37. Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H, Suda T, Shultz LD, Nishikawa S-I. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature (London) **345**:442–444, 1990.
38. Kodama H, Makoto N, Niida S, Yamasaki A. Essential role of macrophage colony stimulating factor in the osteoclast differentiation supported by stromal cells. J Exp Med **173**:1291–1294, 1991.
39. Stein J, Borzillo GV, Rettenmier CW. Direct stimulation of cells expressing receptors for macrophage colony-stimulating factor (CSF-1) by a plasma membrane-bound precursor of human CSF-1. Blood **76**:1308–1315, 1990.
40. Friedenstein AJ, Chailakhjan RK, Latzinik NV, Panasyuk AF, Keiliss-Borok IV. Stromal cells responsible for transferring the microenvironment of the hematopoietic tissues. Transplantation **17**:331–340, 1974.
41. Trentin JJ. Hematopoietic inductive microenvironment. In: Cairne AB, Lala PK, Osmond DG, Eds. Stem Cells. New York: Academic Press, pp255–261, 1979.
42. Dexter TM, Coutinhot LH, Spooncer LCM, Heyworth CP, Danie R, Shiro J, Chang J, Allen TD. Stromal cells in hematopoiesis. In: CIBA Foundation Symposium Staff. Molecular Control of Hematopoiesis: Symposium, No. 148. New York: Wiley, pp76–95, 1990.
43. Allen TD, Dexter TM. Long-term bone marrow cultures: An ultrastructural review. Scanning Electron Microsc **4**:1851–1866, 1983.
44. Gordon MY, Dowding CR, Riley GP, Goldman JM, Greaves MF. Altered adhesive interactions with marrow stroma of hematopoietic progenitor cells in chronic myeloid leukemia. Nature (London) **328**:342–344, 1987.
45. Gordo MY, Riley GP, Greaves MF. Compartmentalization of hematopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. Nature (London) **326**:403–405, 1987.
46. Roberts R, Gallagher J, Spooncer E, Allen TD, Bloomfield F, Dexter TM. Heparan sulphate bound growth factors: A mechanism of stromal mediated hematopoiesis. Nature (London) **332**:376–378, 1988.
47. Zipori D, Sasson T, Frenkel A. Myelopoiesis in the presence of stromal cells from mouse bone marrow. I. Monosaccharides regulate colony formation. Exp Hematol **9**:656–662, 1981.