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Osteocalcin Promotes Differentiation of Osteoclast Progenitors From Murine Long-Term Bone Marrow Cultures

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Abstract Murine long-term bone marrow cultures (LTBMCs) were used to generate hematopoietic cells free from marrow stromal cells. These progenitor cells were treated with GM-CSF (5 U/ml) with or without rat bone osteocalcin or rat serum albumin in either α-MEM with 2% heat-inactivated horse serum alone (α) or supplemented with 10% L-cell-conditioned medium (as a source of M-CSF) (L10). Few substrate-attached cells survived in basal α medium, but when treated with L10 medium or GM-CSF, they survived and proliferated. Osteocalcin did not significantly affect survival or proliferation. Subcultures of cells treated with GM-CSF had large numbers of multinucleated cells, more than half of which were tartrate-resistant acid phosphatase–positive (TRAP). Osteocalcin further promoted the development of TRAP-positive multinucleated cells; a dose of 0.7 μg/ml osteocalcin promoted osteoclastic differentiation by 60%. Using a novel microphotometric assay, we detected significantly more tartrate-resistant acid phosphatase activity in the osteocalcin plus GM-CSF group (75.6 ± 14.2) than in GM-CSF alone (53.3 ± 7.3). In the absence of M-CSF, GM-CSF stimulated tartrate-resistant acid phosphatase activity, but osteocalcin did not have an additional effect. These studies indicate that osteocalcin promotes osteoclastic differentiation of a stromal-free subpopulation of hematopoietic progenitors in the presence of GM-CSF and L-cell-conditioned medium. These results are consistent with the hypothesis that this bone-matrix constituent plays a role in bone resorption.

Key words: osteoclast, osteocalcin, bone marrow, differentiation, resorption

The osteoclast is the multinucleated, matrix-resorbing cell of bone. A variety of in vivo and in vitro systems have been used to study its differentiation. The osteoclast is thought to be derived from a bone marrow hematopoietic mononuclear cell [Ash et al., 1980; Ko and Bernard, 1981], but its exact lineage is unknown. Current evidence suggests that the osteoclast progenitor is related to the monocyte lineage [Burger et al., 1982; Scheven et al., 1986; Kurihara et al., 1989], develops tartrate-resistant acid phosphatase activity [Baron et al., 1986; Takahashi et al., 1988b], develops calcitonin responsiveness [Hattersley and Chambers, 1990], and fuses with other mononuclear cells to form multinucleated osteoclasts [Zambonin-Zallone and Teti, 1985; Scheven et al., 1985]. Several hormones and growth factors have been implicated in osteoclast ontogeny, including 1,25-dihydroxyvitamin D_3 [Sasaki et al., 1989; Roodman et al., 1985; MacDonald et al., 1987], granulocyte-macrophage colony-stimulating factor (GM-CSF) [Kurihara et al., 1989; Hattersley and Chambers, 1990; MacDonald et al., 1986; Lorenzo et al., 1987; Polvony et al., 1990], and macrophage colony stimulating-factor (M-CSF or CSF-1) [Hattersley and Chambers, 1990; MacDonald et al., 1986; Lorenzo et al., 1987; Polvony et al., 1990]. Although emphasis has been on the role of soluble and cell-associated factors in osteoclastogenesis, we have speculated that constituents of the bone matrix may also influence bone resorption [Lian et al., 1984]. Evidence to date from in vivo studies supports the hypothesis that osteocalcin, a bone-specific, calcium-binding protein, functions as a matrix signal for the differentiation of bone-resorbing osteoclasts [Glowacki et al., 1991].

In this study we use an in vitro model of osteoclast differentiation [Liggett et al., 1993] to examine direct effects of osteocalcin. Unique

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features of this system are that it allows dissociation of mitogenic and of differentiating effects and that the absence of bone marrow stromal or osteoblastic cells excludes indirect effects mediated through these cell types. A new method for quantitation of tartrate-resistant acid phosphatase (TRAP) activity in cultured cells was developed to assess this marker of osteoclast activity. We report that, in the presence of M-CSF and GM-CSF, which promote proliferation and differentiation, respectively, of putative progenitors, osteocalcin enhances the expression of osteoclastic features in cultured marrow-derived cells.

METHODS

Continuous Long-Term Bone Marrow Cultures

Long-term bone marrow cultures were established according to published methods [Greenberger, 1978; Sakakeeny and Greenberger, 1982]. Femurs and tibias from 4–12-week-old C57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME) were cleaned of soft tissue. Marrow was flushed from the diaphyses from each mouse with 8 ml of medium through a 23 gauge needle into a 25 cm^2 tissue culture flask (Corning, NY). The medium consisted of Fischer's leukemic medium (GIBCO, Grand Island, NY), 25% horse serum (HS; Hazleton, St. Lenexa, KS), 100 U/ml penicillin (GIBCO), 100 μg/ml streptomycin (GIBCO), and 10^{-5} M hydrocortisone sodium succinate (Abbott Laboratories, North Chicago, IL). The flasks were incubated in a humid environment at 33°C in 5% CO_2 in air. After 4 weeks in culture, the medium was replaced with one containing 25% heat-inactivated fetal bovine serum (FBS; Hazleton, St. Lenexa, KS) instead of horse serum. These primary cultures were maintained for up to 12 weeks.

Media

Basal α medium. α-MEM (GIBCO, Grand Island, NY), 2% heat-inactivated horse serum (HS; Hazleton or GIBCO), 100 U/ml penicillin (GIBCO), and 100 μg/ml streptomycin (GIBCO). Heat-inactivation was accomplished by heating the serum to 56°C for 45 min.

L10 medium. Basal α medium supplemented with 10% L-cell-conditioned medium (as a source of M-CSF). L-929 cells were grown in RPMI (GIBCO) with 2% FBS, 100 U/ml penicillin, and 100 ug/ml streptomycin at 37°C in 5% CO_2 in air. L-cell-conditioned medium was collected from confluent cultures of L-929 cells, centrifuged at 800*g* for 10 min, and stored frozen at −80°C. Experimental cultures were incubated with α or L10 with GM-CSF (recombinant mouse GM-colony stimulating factor) (Genzyme, Boston, MA) and/or osteocalcin. Osteocalcin was purified from rat cortical bone (age 6–8 weeks) that had been defatted in 100% ethanol, dried, and ground to a powder of less than 75 microns. Osteocalcin was extracted as the major protein component in 0.3 N HCl at 5°C for 3 h. The dialyzed and lyophilized extract was reconstituted in trifluoracetic acid buffer for reverse phase C18 chromatography. The osteocalcin fraction was further purified by DEAE ion exchange chromatography obtaining a completely pure preparation of osteocalcin in a single fraction [Glowacki et al., 1991]. Amino acid analysis of the purified component confirmed 3 gla residues in a 49 residue protein. Rat serum albumin was obtained from Sigma (St. Louis, MO).

Differentiation Cultures

The nonadherent cells were collected weekly from long-term bone marrow cultures by gentle aspiration of the supernatant media. The contents from all flasks were pooled, collected by centrifugation at 800*g* for 10 min, and washed twice in α medium. These cells were plated into wells of eight-chamber Permanox® slides (Lab-Tek; Miles Laboratories, Naperville, IL) at a seeding density of 5.2×10^5 cells/cm^2 and incubated for 24 h at 37°C in 5% CO_2 in air (pretreatment incubation). After the pretreatment incubation, the media and nonattached cells were removed by aspiration and fresh basal medium or treatment media were added. Half-media changes were made after 3, 7, and 10 days of culture.

Colorimetric Assay for Cell Number

The nonadherent cells were seeded into 96-well tissue culture plates at 2×10^5 viable cells per well in α medium and were incubated for 24 h at 37°C in 5% CO_2 in air. After this pretreatment incubation, the media and nonattached cells (primarily nonvital granulocytes) were removed by aspiration, and fresh basal medium or treatment medium was added. Half-media changes were made after 3, 7, and 10 days of culture. Cellularity was assessed at 1 or 2 weeks, by the method of Mosman [1983]. The treatment medium was removed and 100 μl of DMEM (GIBCO), and 10% fetal bovine serum (Hazleton), and 10% MTT tetrazolium solution (Chemi-

con) was added per well. The wells were incubated for 3–4 h at 37°C in 5% CO_2 in air. Then 100 μl of the color development solution (isopropanol with 0.04 N HCl) was added, and the contents of each well were mixed by repeated pipetting. Within an hour, the absorbance was measured at 600 nm on a microplate reader (EL308; Bio-Tek Instruments Inc., Winooski, VT).

Group means and standard deviations were analyzed and multiple comparisons were made by one-way analysis of variance. Differences between means were considered statistically significant if $P < 0.05$.

Multinucleation and Tartrate-Resistant Acid Phosphatase (TRAP) Activity

After 14 days in culture, the media were removed and the cells were rinsed three times with PBS followed by fixation with cold acetone: methanol (1:1) for 1 min. The wells were rinsed thoroughly with distilled water following which 400 μl of fresh filtered incubation medium was added to the wells, and the cultures were incubated for 60 min at 37°C. The incubation medium consisted of equal volumes of distilled water and acetate buffer. The acetate buffer contained 5 mg naphthol AS-BI phosphate (Sigma), 0.2 ml dimethylformamide (Sigma), 25 ml of 0.2M acetate buffer, pH 5.2 (Fisher), and 60 mg fast red violet LB salt (Sigma). For assessment of acid phosphatase (AP) and TRAP, respectively, either zero or 5.5 ml tartrate stock (Sigma) was added to the incubation medium followed by 2 drops of 10% manganese chloride (Sigma). After incubation, the wells were washed thoroughly with water and coverslipped with Aquapolymount® (Polysciences Inc., Warrington, PA). The positive cells exhibited pink cytoplasm.

Multinucleation was assessed by counting the number of cells with three or more nuclei in 18 contiguous fields at 20× magnification along a diagonal path from one corner of the chamber to the opposite corner using a 1 mm × 1 mm grid reticle [Liggett et al., 1993]. This is a total area of 4.5 mm^2. An evaluation of total cell number was made in a similar manner. The numbers of multinucleated cells which were either stained and unstained for TRAP or AP were also counted. The percent of total multinucleated cells and the percent of TRAP-positive multinucleated cells were calculated in relation to total cell number and to total multinucleated cells. ANOVA was performed to assess the differences between various groups.

Tartrate-resistant acid phosphatase activity was quantified with a Nikon Phoscan P1 photometer that was coupled to a Nikon light microscope and a Compac® computer. Transmitted light was measured through 40–50 contiguous fields (0.64 mm^2 each) at 4× magnification and expressed as absorbance ($\times 10^3$) per square millimeter. For each chamber, the mean and mean for the most intense 30% were calculated [Glowacki et al., 1991; Liggett et al., 1993]. The thirtieth percentile value is a more robust statistic for describing skew distributions. Data were analyzed and multiple comparisons made by one-way analysis of variance. Differences between means were considered statistically significant if $P < 0.05$.

Contraction Response to Calcitonin

The nonadherent cells were seeded in 25 cm^2 tissue culture flasks (Becton Dickinson & Co., Lincoln Park, NJ) as micromasses (2×10^5 viable cells per micromass) and allowed to preincubate for 24 h at 37°C in 5% CO_2 in air. After the pretreatment incubation, the media and nonattached cells were removed by aspiration, and 6 ml of treatment medium was added. Half-medium changes were made after 3, 7, and 10 days of culture. After 14 days, suitable fields containing both mono- and multinucleated cells were monitored at 37°C with a phase contrast microscope (Diaphot; Nikon), equipped with differential interference optics, a camera (HV 700; Hitachi), and a time-lapse video recorder (Panasonic). After a basal period of 30 min, salmon calcitonin was added at 10^{-9} M. Filming continued for 12 h. Projected areas of cells were measured at 0 and 2 h with a Zeiss ZIDAS digitizing tablet.

RESULTS

Effect of Osteocalcin on Cellularity

Nonadherent cells from long-term bone marrow cultures were incubated in 96-well tissue culture plates for 24 h in α medium. The attached cells were subsequently treated with or without GM-CSF (5 U/ml) or osteocalcin (Oc) at 5 different concentrations for 1 week. Few cells receiving α or α with osteocalcin for 1 week survived. In contrast, treatment with α and GM-CSF caused a significant increase ($P < 0.05$) in cellularity compared to α alone (Fig. 1A).

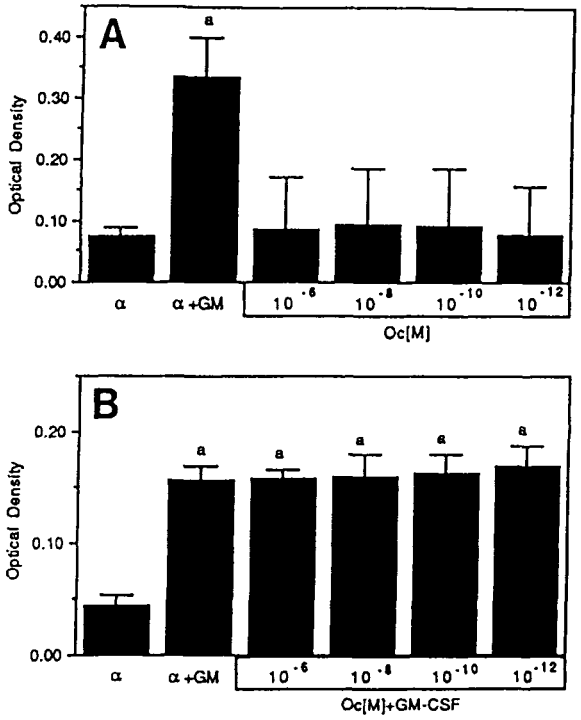

Fig. 1. Cell density determined by colorimetric assay. **A:** Effect of GM-CSF (5 U/ml) or osteocalcin (Oc) after 1 week. Each bar represents the mean ± S.D. for four determinations. **B:** GM-CSF (5 U/ml) was added for both weeks, and osteocalcin (Oc) was added at different concentrations during the second week. Each bar represents the mean ± S.D. for six determinations. a, indicates different from α ($P < 0.05$).

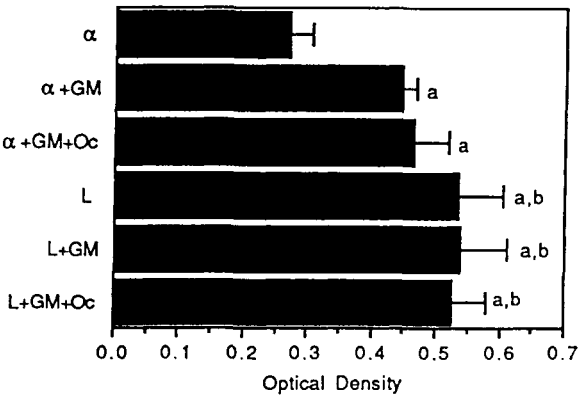

Fig. 2. Cell density determined by colorimetric assay. GM-CSF (5 U/ml) and osteocalcin (Oc) (1.2×10^{-6} M) were added to α or L10 basal media. Each bar represents the mean ± S.D. for five determinations. a, indicates different from α. b, indicates different from α + GM ($P < 0.05$).

Supplementation with different concentrations of osteocalcin did not alter cellularity in α medium.

Other cultures were treated with GM-CSF for 2 weeks with various concentrations of osteocalcin added during the second week. Phase microscopic observations of α-treated cultures revealed fewer cells at 2 weeks than at 1 week, consistent with the poor supportive capacity of this medium. Treatment with GM-CSF caused a significant increase ($P < 0.05$) in cellularity (Fig. 1B). Addition of osteocalcin did not alter the stimulatory effect of GM-CSF.

The influence of 2 weeks treatment with osteocalcin was assessed in α medium compared to L10 medium. As above, treatment with GM-CSF in α significantly increased cellularity ($P < 0.05$) (Fig. 2). Cellularity with GM-CSF was similar with or without 2 weeks treatment with 1.2×10^{-6} M osteocalcin. Cellularity was greater with L10 or L10 with GM-CSF than with α and GM-CSF (Fig. 2). Treatment with L10 did not differ from L10 with GM-CSF. Thus, under these conditions of low serum supplementation, maximal proliferation can be achieved with 10% L-cell-conditioned medium. Treatment with osteocalcin for 2 weeks did not affect proliferation in the presence of L10 and GM-CSF. These conditions were therefore suitable for examining differentiation in cultures with similar cell densities.

Effect of Osteocalcin on Multinucleation

The effects of GM-CSF, L-cell-conditioned medium, and osteocalcin on multinuclearity were examined. Because cell density was not uniform within the wells but appeared greatest along the periphery of each well, multinuclearity was assessed by counting the number of cells with three or more nuclei in 18 contiguous fields along a diagonal path from one corner of the chamber to the opposite corner. This method produced data representative of the entire chamber [Liggett et al., 1992]. In α medium, cells failed to proliferate well, and there were few (10 ± 4) multinucleated cells per sample (Table I). Cells treated with GM-CSF in α medium developed elevenfold more multinucleated cells than in α medium alone ($P < 0.05$). Osteocalcin did not significantly alter the stimulation of multinuclearity by GM-CSF. The result was corroborated by a repeated experiment.

Cultures treated with L10 and GM-CSF developed up to 4.3-fold more multinucleated cells in comparison to L10 treatment. Taken together with the fact that cell densities were similar with L10 or L10 plus GM-CSF (Fig. 2), GM-CSF's effect on multinuclearity cannot be explained solely on the basis of cellularity (Table

TABLE I. Effect of Osteocalcin on the Development of Multinucleated Cells†

System	MNC/well x ± S.D.
α system	
α	10 ± 4
α + GM (5 U/ml)	110 ± 69*
α + GM + Oc (7 μg/ml)	170 ± 92*
L10 system	
L10	36 ± 9
L10 + Oc (0.07 μg/ml)	26 ± 9
L10 + Oc (0.7)	29 ± 8
L10 + Oc (7)	48 ± 16
L10 + GM	153 ± 15**
L10 + GM + Oc (0.07)	156 ± 28**
L10 + GM + Oc (0.7)	187 ± 27**
L10 + GM + Oc (7)	212 ± 5***
L10 + RA (0.07)	39 ± 7
L10 + RA (0.7)	41 ± 17
L10 + RA (7)	47 ± 7
L10 + GM + RA (0.07)	145 ± 12**
L10 + GM + RA (0.7)	158 ± 32**
L10 + GM + RA (7)	154 ± 25**

†Multinucleated cells with three or more nuclei were counted and expressed as the mean (x) ± standard deviation for four wells. The α system included basal medium with 2% heat-inactivated horse serum; the L10 system was α medium supplemented with 10% L-cell-conditioned medium as a source of M-CSF. GM indicates GM-CSF, Oc indicates osteocalcin, and RA indicates rat serum albumin. Cells were treated for 2 weeks.
*Significant vs. α at $P < 0.05$.
**Significant vs. L10 at $P < 0.05$.
***Significant vs. L10 and L10 + GM at $P < 0.05$.

I). Cells treated with L10 and osteocalcin did not show significant changes in multinucleation vs. L10 treatment. This result was corroborated by two additional repeat experiments. In one experiment, 7 μg/ml osteocalcin caused a small (13%) but significant enhancement of multinuclearity above the GM-CSF effect (Table I). The data suggest that osteocalcin does not have a significant, reproducible effect on total multinuclearity. As a protein control for rat bone osteocalcin, rat albumin had no effect on total multinuclearity.

Effect of Osteocalcin on Osteoclastogenesis

After a 2 week treatment period, cultures were stained for tartrate-resistant acid phosphatase (TRAP) activity without counterstain. Total TRAP activity was quantified by a new microphotometric technique that is based upon the reduction of light transmitted through stained cultures [Glowacki et al., 1991; Liggett et al., 1993]. Transmitted light was measured through 40–50 contiguous fields (0.64 mm^2 each) at 4× magnification and expressed as absorbance ($\times 10^3$) per square millimeter. For each chamber, the mean of all values and mean for the most intense 30% were calculated. Using the means of the thirtieth percentiles avoided the nonparametric skewing by fields of unstained cells that were especially common in the basal α and L10 media.

In α medium, treatment with GM-CSF significantly increased TRAP activity twofold (Table II). Cells treated with GM-CSF and osteocalcin showed no additional response. Cells treated with L10 and GM-CSF had significantly greater TRAP activity than cells treated with L10 media. The activity was 35–40% higher after L10 and GM-CSF treatment in two experiments compared to L10 treatment. As was the case for multinuclearity, GM-CSF's effect on TRAP activity is a differentiation effect not linked to proliferation. TRAP activity in cells treated with L10 and osteocalcin did not differ significantly from cells treated with L10. However, cells that received L10 with GM-CSF and osteocalcin had 30% more activity compared to cells treated with L10 and GM-CSF (Fig. 3). Osteocalcin did not enhance tartrate-resistant acid phosphatase activity in α medium (i.e., in the absence of M-CSF).

In a more extensive study, we examined the effects of various concentrations of rat bone osteocalcin and rat albumin on cellularity and on development of osteoclastic, TRAP-positive multinucleated cells (Table III). Rat albumin served as a protein control and failed to show significant effects on these parameters either in the presence or absence of GM-CSF. GM-CSF promoted the development of TRAP-positive multinucleated cells with 56% of the total number of MNCs positive for the enzyme, compared to only 28% of the smaller number of MNCs in L10. In a dose-dependent manner, osteocalcin promoted the formation of TRAP-positive MNCs and the proportion of TRAP-positive MNCs, but only in the presence of GM-CSF (Fig. 4). At 0.7 μg/ml, osteocalcin increased the number of TRAP-positive MNCs by 60% ($P < 0.01$); at 7.0 μg/ml, stimulation was 84% ($P < 0.01$). A critical feature of these experiments is that cellularity was not significantly different in the treatment groups; thus, effects on multinucleation and differentiation were examined with similar cell densities.

In a study to confirm that the TRAP-positive multinucleated cells were osteoclast-like, calcito-

TABLE II. Effect of GM-CSF, L-Cell-Conditioned Medium, and Osteocalcin on Tartrate-Resistant Acid Phosphatase (TRAP) Activity†

System	TRAP activity (abs. × 10^3 per mm^2) x ± S.D.	x thirtieth percentile ± S.D.
α system		
α	13.45 ± 1.60	20.93 ± 1.95
α + GM (5 U/ml)	25.35 ± 4.08*	45.77 ± 7.31*
α + GM + Oc (7 μg/ml)	22.24 ± 5.63*	26.41 ± 6.10
L10 system		
L10	24.70 ± 2.49	39.06 ± 5.46
L10 + GM (5 U/ml)	33.49 ± 2.28**	53.23 ± 7.39**
L10 + Oc (7 μg/ml)	23.54 ± 4.54	37.53 ± 13.69
L10 + GM + Oc (7 μg/ml)	43.78 ± 9.72***	75.68 ± 14.18***

†TRAP activity was assessed microphotometrically and expressed as the mean (x) and mean of the thirtieth percentile ± standard deviation for four wells. The α system included basal α-MEM with 2% heat-inactivated horse serum; the L10 system was supplemented with 10% L-cell-conditioned medium as a source of M-CSF. GM indicates GM-CSF and Oc indicates osteocalcin.
*Significant vs. α at $P < 0.05$.
**Significant vs. L10 at $P < 0.05$.
***Significant vs. L10 and L10 + GM at $P < 0.05$.

nin responsiveness of these putative osteoclasts was evaluated by seeding progenitor cells in tissue culture flasks in α medium, incubating for 24 h, and treating the cells in L10 with GM-CSF and 1.2×10^{-6} M osteocalcin for 2 weeks. A subpopulation of the large multinucleated cells contracted 0.5 h after treatment with calcitonin. In randomly selected multinucleated cells, the projected area of each cell decreased to 52 ± 12% of their baseline areas. The mononuclear cells did not change after treatment. Thus, it is demonstrated that these cells show the osteoclastic response to calcitonin rather than the phenotype of the macrophage polykaryon.

DISCUSSION

Previously, we reported that L-cell-conditioned medium (as a source of M-CSF), GM-CSF, and IL-3 are mitogenic for these osteoclast progenitors; GM-CSF and IL-3, but not M-CSF, promote development of TRAP-positive multinucleated cells [Liggett et al., 1993]. In this

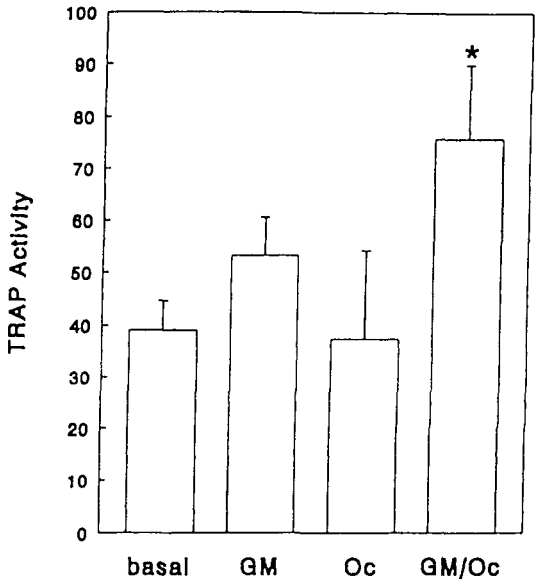

Fig. 3. Effect of osteocalcin (Oc) on tartrate-resistant acid phosphatase (TRAP) activity in medium containing 10% L-cell-conditioned medium without or with 5 U/ml GM-CSF. Total TRAP activity was assessed microphotometrically and expressed as the mean ± S.D. for three determinations. *indicates significantly different from all other groups.

study, we found that, when combined with both M-CSF and GM-CSF, osteocalcin further stimulates their differentiation.

Recent investigations on in vitro differentiation of osteoclasts have focused on the role of calcitropic hormones and cytokines on whole or fractionated marrow. The studies reported herein differ in significant ways from other culture systems. This system utilized stromal cell–free populations. Because mature macrophages, megakaryocytes, and stromal cells may have been present in the fractionated compartment of the cells studied by others [Takahashi et al., 1988a; Sasaki et al., 1989; Roodman et al., 1985; MacDonald et al., 1987], it is important to note that cells from long-term bone marrow cultures were devoid of these cells. The target cells in our studies were treated in media containing minimal amounts of serum (2%), in contrast to the greater amounts of serum used by Takahashi et al. (10%) and MacDonald et al. (20%). Furthermore, in contrast to prior reports, we assayed the cellularity of treated cells to assess the degree of cellular proliferation with each treatment. In this manner, it is possible to identify factors that have major effects on proliferation and to control for this important variable. We previously determined that 10% L-cell-conditioned medium, 5 U/ml GM-CSF, and 2% horse

TABLE III. Effects of Rat Bone Osteocalcin (Oc) or Rat Serum Albumin (RA) on Cellularity and TRAP-Positive Multinucleated Cells or (TRAP^{+ve}) After 2 Weeks Culture in L10 Medium With or Without 5 U/ml GM-CSF†

Group	Total cells/ 4.5 mm^2	% TRAP^{+ve} MNCs/ total cells
L	1,520 ± 54	0.71 ± 0.56
L + Oc (0.07 μg/ml)	1,589 ± 27	0.89 ± 0.56
L + Oc (0.7)	1,519 ± 36	0.76 ± 0.49
L + Oc (7)	1,604 ± 26	0.83 ± 0.67
L + GM	1,643 ± 37	5.24 ± 0.42*
L + GM + Oc (0.07)	1,614 ± 7	5.38 ± 0.60*
L + GM + Oc (0.7)	1,625 ± 22	8.36 ± 1.23**
L + GM + Oc (7)	1,613 ± 14	9.62 ± 0.42**
L + RA (0.07)	1,602 ± 37	0.68 ± 0.51
L + RA (0.7)	1,608 ± 15	0.65 ± 0.49
L + RA (7)	1,627 ± 26	0.47 ± 0.48
L + GM + RA (0.07)	1,621 ± 31	5.28 ± 0.43*
L + GM + RA (0.7)	1,623 ± 24	5.72 ± 1.53*
L + GM + RA (7)	1,612 ± 10	5.89 ± 0.65*

†L indicates basal L10 medium; GM indicates 5 U/ml GM-CSF. Each value represents the mean ± standard deviation of four samples. TRAP^{+ve} indicates TRAP-positive multinucleated cells.
*Significant vs. L10 at $P < 0.05$.
**Significant vs. L10 and L10 + GM at $P < 0.05$.

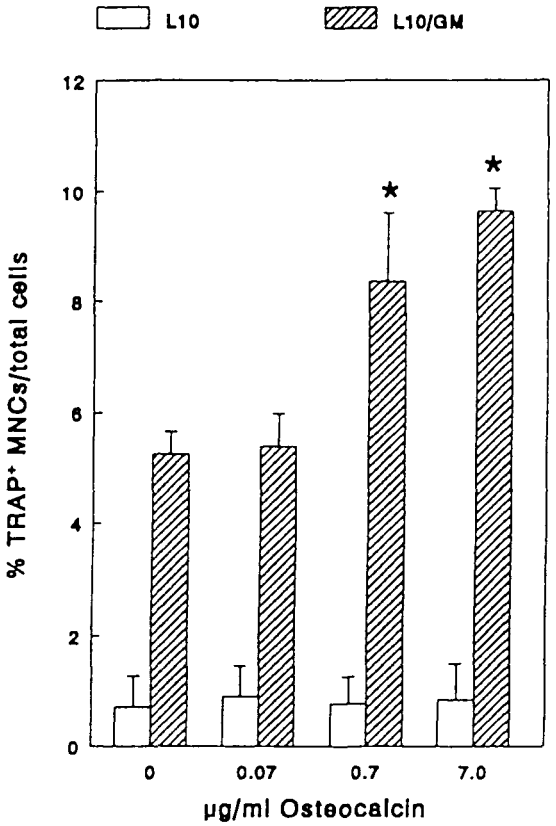

Fig. 4. Effect of osteocalcin on development of TRAP-positive multinucleated cells in medium supplemented with 10% L-cell-conditioned medium (L10) and GM-CSF (L10/GM). TRAP-positive MNCs are expressed as the percentage of total cell number, mean ± S.D. of four replicates. *indicates significantly different from L10/GM, $P < 0.05$.

serum provided optimal conditions for proliferation of these cells [Liggett et al., 1993]. Another difference is that we evaluated differentiation after 2 weeks, whereas those studies that reported effects after 3 or more weeks may include secondary actions, possibly mediated through stromal cells [Shevde et al., 1994]. Finally, in our studies, osteoclast features were assessed by TRAP-positive multinucleated cell numbers, TRAP activity measured by a new photometric technique, and calcitonin responsiveness.

The cell density of the cultures was maximal under these experimental conditions, consistent with the mitogenic activity of L-cell-conditioned medium or GM-CSF [Metcalf, 1986; Sieff, 1987]. Osteocalcin did not act as a mitogen in this system. Kandemir et al. [1990] reported that 10^{-12} M osteocalcin augmented murine marrow colony formation and osteoclastogenesis in marrow cocultured with fetal mouse metatarsal bones, but the mechanism of that effect is not known.

Multinuclearity increased after treatment with L-cell-conditioned medium, with GM-CSF, and with GM-CSF and L-cell-conditioned medium. These data are consistent with the work of MacDonald et al. [1986] who found that GM-CSF or CSF-1 increased multinucleation of cultured baboon bone marrow cells in comparison to control cultures receiving media alone for 3 weeks. On the other hand, Sutton and Weiss [1966] observed that macrophages fuse in vitro to form multinucleated cells, and Abe et al. [1983] generated multinucleated cells from mouse alveolar macrophages by treatment with 1,25-dihydroxyvitamin D_3. Thus, the process of cell fusion leading to multinuclearity is not a specific marker for osteoclast differentiation but may indicate the density-dependent fusion of macrophages. Osteocalcin alone had no effect on cellularity but may have had a small, inconsistent effect on multinucleation. These data clearly show that, with these progenitors, two populations of multinucleated cells developed under these conditions: an osteoclastic population (TRAP-positive) and a macrophage polykaryon population. With osteocalcin treatment, more osteoclastic cells developed with more intense tartrate-resistant acid phosphatase activity. Thus, we

propose that osteocalcin is not the sole stimulus of osteoclastogenesis but can be assigned a role as an enhancer under conditions in which appropriate mitogens and early differentiation signals are active; GM-CSF and M-CSF are two agents that provide these other signals. We have reported one other factor that appears to operate as an enhancer in this system; in the presence of GM-CSF and M-CSF, increasing doses of interleukin-6 increased multinuclearity, the percentage of TRAP-positive multinucleated cells, and total TRAP activity [Shevde and Glowacki, 1992].

Controversies exist on the relative importance of putative differentiation factors, mediators, and helper cells, in part because a specific marker for osteoclasts has yet to be developed. Our studies use markers that are appropriate for murine cells. Tartrate-resistant acid phosphatase (TRAP) [Kurihara et al., 1989; Takahashi et al., 1988b; Sasaki et al., 1989; MacDonald et al., 1987; Polvony et al., 1990; Van de Wijngaert et al., 1987; Helfrich et al., 1989], multinucleation [Ko and Bernard, 1981; Kurihara et al., 1989; Takahashi et al., 1988a; Sasaki et al., 1989; Roodman et al., 1985; MacDonald et al., 1986, 1987], calcitonin responsiveness [Hattersley and Chambers, 1989, 1990], ultrastructure [Ko and Bernard, 1981; Sasaki et al., 1989; MacDonald et al., 1987], and resorption of slices of bone or dentine [Takahashi et al., 1988b; Hattersley and Chambers, 1990] have been used to identify osteoclast-like cells in vitro. In murine systems, Takahashi et al. [1988b] reported that mouse peripheral blood mononuclear cells, peritoneal macrophages, alveolar macrophages, and spleen cells do not develop TRAP activity in vitro. Sasaki et al. [1989] observed the absence of TRAP activity in cultures of mouse alveolar macrophages that developed multinucleated cells and Wijngaert et al. [1987] found that mouse peritoneal macrophage cytospin preparations were TRAP-negative. Helfrich et al. [1989] observed that freshly isolated mouse peripheral blood leukocytes were negative for TRAP activity and remained so after 2 weeks of culture. On the other hand, Kuehler et al. [1981] observed that freshly isolated mouse peripheral blood leukocytes were negative for TRAP activity but found that after 3 weeks in vitro these cells expressed TRAP activity. Furthermore, Hattersley and Chambers [1990] found that mouse peritoneal macrophages which were initially TRAP-negative became TRAP-positive after 7 days of incubation. Nevertheless, the weight of the evidence to date suggests that TRAP activity, while not a specific marker for osteoclasts in vitro, may be more useful as a screen for mouse than for human osteoclasts.

In vitro, cellular responsiveness to calcitonin has been used to identify osteoclasts, assessed by striking cell-shape changes [Chambers and Magnus, 1982] or by specific binding of radiolabeled calcitonin [Van de Wijngaert et al., 1987; Hattersley and Chambers, 1989; Nicholson et al., 1986; Takahashi et al., 1988a]. Our demonstration of cellular contraction in response to calcitonin is consistent with the osteoclastic phenotype. Ultrastructural identification of clear zones and ruffled borders was made when these cells were cultured on bone fragments with GM-CSF and M-CSF [Liggett et al., 1993].

Recent in vivo studies lend support to the hypothesis that osteocalcin acts in concert with other factors to regulate various stages of osteoclastogenesis. Osteocalcin is a noncollagenous bone specific protein accumulated in the extracellular matrix and binds avidly to bone mineral via its calcium-binding γ-carboxyglutamic acid (gla) residues. In warfarin-treated animals, vitamin K–dependent posttranslational synthesis of the gla residues is inhibited, thereby producing an osteocalcin-deficient bone matrix. We reported that osteoclastic differentiation was impaired in response to such bone particles, virtually devoid of osteocalcin [Lian et al., 1984]. Whereas the subcutaneous implantation of devitalized mineral-containing bone particles elicits the recruitment and differentiation of multinucleated cells that resorb bone matrix, a 50% decrease in the number of multinucleated cells was observed with implantation of osteocalcin-deficient bone particles. In addition, overall cellular recruitment was depressed to 35% of control [Glowacki and Lian, 1987]. These effects are highly specific; mixtures of normal and osteocalcin-deficient bone particles (with one or the other type prelabeled with tetracycline) demonstrated that activated osteoclasts were associated only with the normal bone particles but not the adjacent osteocalcin-deficient particles [DeFranco et al., 1991]. The suggestion that osteocalcin functions as a matrix signal in osteoclast development was further tested by comparing the cellular responses to synthetic particles of bone-like apatite with or without osteocalcin. Pure apatite particles elicited generation of foreign body giant cells, but particles containing 0.1% osteocal-

cin were partially resorbed and generated more multinucleated, cells that expressed TRAP activity and ultrastructural features of osteoclasts [Glowacki et al., 1991]. These studies suggested that osteocalcin functions as an insoluble substrate signal for osteoclastogenesis. This may explain why low concentrations of osteocalcin solutions were not effective in the cultures described in this report.

Several possibilities exist for the action of *osteocalcin*. It may potentiate the effect of GM-CSF and direct myeloid cell development toward TRAP-positive progeny by directly binding to hematopoietic progenitor cells. Alternatively, because GM-CSF has been shown to bind to glycosaminoglycans [Gordon et al., 1987], including heparan sulphate [Roberts et al., 1988], osteocalcin may increase differentiating effects of GM-CSF by binding to GM-CSF and making GM-CSF more competitive for binding to hematopoietic cells than M-CSF. Osteocalcin may inhibit differentiation of the macrophage lineage, leaving more target cells responsive to GM-CSF's differentiation effects.

These studies confirm our previous findings that a subpopulation of hematopoietic progenitor cells from murine long-term bone marrow cultures includes the osteoclast progenitor and that its survival, proliferation, and differentiation is stimulated by GM-CSF and M-CSF [Liggett et al., 1993]. Further stimulation of differentiation was achieved by supplementation with osteocalcin. It should be emphasized that these effects were demonstrated in populations of cells enriched for macrophage/granulocyte progenitors and lacking stromal cells. Other regulatory processes can be mediated through marrow stromal cells [Shevde et al., 1994]. These results are consistent with the hypothesis that osteocalcin, the bone matrix constituent, may be an organ-specific signal that plays a role in the differentiation of the bone-resorbing osteoclasts.

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