Identification and Characterization of Osteoclast-like Cells and Their Progenitors in Cultures of Feline Marrow Mononuclear Cells

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ABSTRACT The predominant cell responsible for bone resorption, the multinucleated osteoclast, has been difficult to study because of inaccessibility. When feline marrow-derived mononuclear cells are established in long-term culture, multinucleated cells form within 48 h, reaching maximum numbers at 16 d. We have observed that these cultured cells have many of the features of osteoclasts. Morphologically, they are multinucleated, contain large numbers of branched mitochondria, have a peripheral cytoplasm lacking organelles (a clear zone), and have extensive cell-surface processes. In addition to these ultrastructural features, the cells contain a tartrate-resistant acid phosphatase, the activity of which is increased by parathyroid hormone (PTH) and inhibited by calcitonin. PTH, prostaglandin E2, and 1,25(OH)2 vitamin D3 increased multinucleated cell formation, while calcitonin inhibited the stimulatory effects of PTH. Time-lapse cinemicrographic and autoradiographic studies indicated that the multinucleated cells formed by fusion of the mononuclear progenitors. The multinucleated cells were phagocytic and stained with nonspecific esterase, consistent with their being derived from immature monocytes. Further, cell populations enriched for multinucleated cells release 45Ca from devitalized bone. Density-gradient centrifugation on Percoll was used to enrich and characterize the mononuclear progenitors of these multinucleated cells. The progenitor cells were found predominantly in Percoll density layers of 1.065 to 1.08 g/ml and were enriched up to 30-fold as compared to unfractionated cells. The bone marrow mononuclear cells that formed the multinucleated cells were initially nonadherent to plastic, stained heavily with nonspecific esterase, and appeared to be immature monocytes histologically. These data indicate that the multinucleated osteoclast-like cells in our cultures are derived from nonadherent mononuclear progenitor cells that are responsive to osteotropic hormones. The ability to grow and characterize these cells in vitro should facilitate studies to elucidate the role these cells play in normal and pathologic states of bone resorption.

Studies using a variety of model systems including quail-chick chimeras, parabiotic rats, and bone marrow transplantation have shown that osteoclast precursors are present in hematopoietic tissues, and have suggested that osteoclasts result from fusion of mononuclear phagocytes (1-6). Several in vitro studies have shown that mononuclear phagocytes such as peripheral blood monocytes can form multinucleated giant cells, but these multinucleated cells differ ultrastructurally from osteoclasts (7, 8).

Although some investigators have described cells with osteoclast-like features in cultures isolated from rodent calvaria (9, 10), these cells do not resemble osteoclasts morphologically. Technical difficulties have hampered attempts to obtain a homogeneous, readily identifiable osteoclast population from adult mammals and have been a major limitation in understanding the pathophysiology of age-related bone loss and other metabolic bone diseases.

Recently, Testa et al. (11) described a technique in which they established feline bone marrow cells in long-term culture. These investigators observed multinucleated cells with some
of the morphological characteristics of osteoclasts in their cultures. These cells were multinucleated, survived for several months in vitro, ultrastructurally had a clear zone with adjacent ruffled borders, and showed increased numbers of nuclei per cell with continued culture. However, the evidence that these cells were osteoclasts was incomplete, since these cells were not tested for their capacity to resorb bone or respond to osteotropic hormones. In the current investigation, similar

MATERIALS AND METHODS

Cell Culture

Cat marrow cells were cultured as described by Testa et al. Briefly, 12-16 wk-old female cats were killed by intracardiac injection of sodium methohexitol. The femurs were disarticulated, the ends removed, and the bone marrow cells were flushed out with a syringe fitted with an 18 gauge needle containing alpha-minimal essential medium (alpha-MEM) (Gibco Laboratories, Grand Island, NY). A single cell suspension was prepared by repeated pipetting with a 10-ml pipette. In selected experiments, unfractionated bone marrow cells were incubated for 2 h in alpha-MEM plus 30% horse serum at 37°C in plastic tissue culture flasks, and the nonadherent cells collected. The nonadherent and adherent cells were then cultured separately in alpha-MEM with 30% horse serum. All cultures were observed daily and the number of multinucleated cells in 20 microscopic fields at a final magnification of 250 was counted using an inverted phase-contrast microscope. The cells were cultured at a final density of 10^5 cells/ml in alpha-MEM containing 30% (vol/vol) horse serum (Flow Laboratories, McLean, VA) and 500 U/ml penicillin and 50 ag/ml streptomycin (Gibco Laboratories) in either 25-cm² flasks (Falcon Labware, Oxnard, CA), 6-well (35-mm diameter) or a 24-well (16-mm diameter) plates (Linbro, Flow Laboratories, McLean, VA), and incubated at 37°C in a humid 5% CO₂-air atmosphere. The cultures were fed weekly by removing half of the medium and replacing it with fresh medium. Cells removed with the spent medium were not replaced.

Histology and Ultrastructural Studies

 Cultures were examined by phase-contrast microscopy at × 40 and × 200. For histologic studies on bone marrow cultures, the culture medium was removed and the adherent cells fixed in situ with 5% glutaraldehyde for 5 min. The cells were washed with water, allowed to air dry, and then stained with Wright’s Giemsa stain or hematoxylin. In selected experiments, marrow cultures were allowed to air dry before fixation and then stained with either periodic-acid Schiff stain, alpha-naphthyl-ASD-butyrate (nonspecific esterase) or naphthol ASD chloroacetate (granulocytic esterase) by standard techniques (12). Marrow cultures were then examined by light microscopy. Other cultures were stained for acid phosphatase activity as described below. Cytospin slides were prepared by placing 0.1 ml of unfractionated bone marrow cells or cells from each layer of the gradient (2 × 10⁶ cells/ml) in a cytocentrifuge (Shandon Southern Instruments, St. Sewickley, PA) followed by centrifugation at 1,000 rpm for 10 min to transfer the cells to glass slides. Slides were air dried, fixed with methanol, and stained with Wright’s Giemsa stain, and a differential cell count done. 200 cells were counted in each specimen.

For ultrastructural studies, cells grown on plastic plates were fixed in modified Karnovsky’s solution (13) containing 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, 320 mosmol for 90 min at 4°C. After washing and additional fixation in 1% osmium tetroxide (60 min at 4°C), the cells were washed again, then stained en bloc with aqueous uranyl acetate prior to embedding in Embed 812 (Polysciences, Inc., Niles, IL) araldite. Ultrathin sections were counterstained in lead acetate and uranyl acetate before viewing in a Philips 301 electron microscope at an operating voltage of 60 kV. For studies with bone particles present in the cultures (see below), cultures were decalcified with EDTA (250 mM, pH 6.8) for 1 h after fixation and then processed as described above.

Acid Phosphatase Determinations

The effect of osteotropic hormones on acid phosphatase activity was determined after cells had been grown for 2 wk in six-well plates (Linbro). Bovine PTH (200 ng/ml), calcitonin (100 ng/ml), or PTH and calcitonin were added to the cells and the cultures continued an additional 24 h. PTH (1-84) was obtained from the National Pituitary Agency (sp. act. 1,300 IU/mg protein). Salmon calcitonin (Calcimar T.M.) was obtained from the Armour Pharmaceutical Co. (Phoenix, AZ). The medium was aspirated and the cells washed twice with phosphate-buffered saline. The cells in each well were then fixed for 30 seconds in 1 ml citrate-buffered acetone (0.15 M sodium citrate, pH 5.4, in 60% (vol/vol) acetone). The cells were then rinsed in deionized water and stained (1 ml per well) for acid phosphatase activity for 1 h at 37°C in the dark using a histochemical kit (Sigma Chemical Co.). The acid phosphatase staining reaction involves the enzymatic hydrolysis at acid pH of a naphthol AS-BI phosphoric acid substrate to yield a naphthol AS-BI which instantly couples with a benzidine GBC in the mixture to form an insoluble magenta dye at the sites of enzyme activity. Because the acid phosphatase activity of the osteoclast is insensitive to inhibition by tartrate (14), we added sodium tartrate (2x 10⁻⁴ M) to some of the culture wells simultaneously with the acid phosphatase staining mixture. The cells were then rinsed in deionized water, and counter stained with acid hematoxylin (pH 3.3) for 5 min, rinsed with deionized water for 3 min and allowed to air dry. All concentrations of the hormone were tested in triplicate. Cells were scored for acid phosphatase activity (staining density) by counting 20 microscopic fields at ×1,000 in each culture. Scoring was done without knowledge of treatment group. At least 20 multinucleated cells were counted at random within each well.

TIME-LAPSE CINEMICROGRAPHY:

After 1 wk of culture, 200 ng/ml PTH was added to the marrow cultures and the cultures placed at 37°C in an incubation chamber fitted to an inverted microscope (Nikon Instruments, Garden City, NY) equipped with a Bolex H-16 cine camera. Exposures were taken every 30 s for 18 h. Time-lapse studies were done on four separate experiments.

AUTORADIOGRAPHY:

Cells were cultured for 1 wk as described above. 5 μCi/ml [3H]hydroxyde (30 Ci/mmol [ICH, Irvine, CA]) was added to each of the cultures, and the cultures incubated for 24 h. The cultures were then washed extensively with alpha-MEM 30% horse serum. 200 ng/ml PTH was then added to the cultures and the cultures incubated for 18 h at 37°C. The cultures were then processed for autoradiography as described by Roodman et al. (15).

ENRICHMENT OF OSTEOCLAST PRECURSORS:

Percol density gradients were prepared by diluting Percol (Pharmacia Fine Chemicals, Uppsala, Sweden) with 1.5 M sodium chloride (9:1). This stock Percol was further diluted with 0.15 M sodium chloride to give a density range of 1.05-1.10 g/ml. The density of each layer was calculated from the refractive index. Ten 1-ml fractions were layered in conical 15-ml tubes, and 3 × 10⁶ feline marrow cells suspended in 3 ml alpha-MEM were layered over the top of the gradient. These heterogeneous gradients were centrifuged at 400 × g for 30 min at 4°C, and the cells at the interfaces collected. The cells were washed twice with alpha-MEM and a nucleated cell count done. Cytospin slides for histology were prepared from each fraction as described above. The cells in each fraction were then cultured separately, Unfractionated bone marrow cells (controls) or cells from each Percol density layer were cultured in alpha-MEM 30% (vol/vol) horse serum at a density of 10⁶ cells/ml in 25-cm² flasks. Cultures were examined for periodic-acid Schiff staining, nonspecific esterase or granulocytic esterase staining as described above.

Bone Resorption

Fractionated and nonfractionated bone marrow cells were cultured in 24 well plates (Linbro) at 10⁶ cells/ml per well or were fractionated on Percoll density gradients as described above. After 2 wk in culture, supernatant medium was removed and diluted by 50% with fresh medium. PTH (200 ng/ml),
calcitonin (100 ng/ml), or PTH and calcitonin were added, and the medium added back to the cells. Devitalized bone particles (±25 μM diameter) pretreated with 45Ca (16) (kindly provided by Drs. S. L. Teitelbaum and Z. Bar-Shavit) and sterilized by ultraviolet irradiation overnight were added to each culture well, (0.11 mg/well). The cells were incubated for 48 h at 37°C in a humid atmosphere of 5% CO2-air. To determine the amount of 45Ca released from the bone particles into the medium. 0.2-ml aliquots of medium were removed and counted in a liquid scintillation counter. Trichloroacetic acid (100% wt/vol) was then added to the cultures (0.1 ml/well) and the cultures incubated for 24 h to solubilize the remaining bone particles. The medium was then aspirated from the wells and counted in a scintillation counter. Results are expressed as percent release of total 45Ca into the culture medium during the culture period.

RESULTS

Multinucleated cells (Fig. 1) began to appear in significant numbers in unfractionated feline marrow cultures after 2 d (Table I). Maximum numbers of multinucleated cells were found after 16 d of culture and remained stable until day 24 of culture. Such cultures have been maintained for more than 6 wk. Ultrastructurally, the nuclei of these cells were uniform with prominent nucleoli and a minimum of heterochromatin, which was displayed as a thin band at the nuclear periphery. Abundant cytoplasmic organelles were present and included lipid droplets, free ribosomes (both singly and as polyribosomes), rough endoplasmic reticulum, and pleomorphic mitochondria (Fig. 2). In most cells, areas of peripheral cytoplasm (clear zones) that lacked organelles, other than free ribosomes were identified and circumferential bundles of microfilaments were present at the cell surface in these areas (Fig. 2). Contractile apparatuses, both microtubules and microfilaments, also were distributed throughout the cytoplasm of these cells. Other cell surface specializations included extensive cytoplasmic membrane foldings (Fig. 3B). Associated with these folded cell edges were focal areas of thickened cell membranes which resembled coated pits and which occasionally appeared as internalized or intracellular structures (Fig. 3C). Also associated with these areas of the cells were numerous vacuoles, some of which contained amorphous granular material (Fig. 3A). At least two distinct types of monocyted cells were regularly observed in the 14-d-old feline marrow cultures. One type (Fig. 4) contained lipid droplets, a subplasmalemmal band of microfilaments and a single nonlobulated nucleus with a prominent nucleolus and a peripheral rim of condensed chromatin; these features are consistent with the ultrastructural characteristics of the multinucleated cells. The other monocyted cells were typical monocytes with amorphous granules, small but uniform mitochondria, and a single lobed nucleus (Fig. 5).

The effects of osteotropic hormones on multinucleated cell formation was then examined. Addition of PTH (200 ng/ml) (Table II) increased the number of multinucleated cells twofold as compared to untreated control cultures, while calcitonin (100 ng/ml) alone had no significant effect on multinucleated cell formation. However, addition of calcitonin blocked the stimulatory effects of PTH on multinucleated cell formation. In addition, addition of vitamin D3, substances which stimulate osteoclast formation in vivo, also significantly increased the number of multinucleated cells (Table III).

PTH and calcitonin also affected acid phosphatase activity of the multinucleated cells (Table IV). PTH stimulated acid phosphatase activity approximately twofold, and this increased enzyme activity was not inhibited in the presence of 27 mM sodium tartrate. Tartrate appeared to cause a slight but significant increase in PTH stimulated acid phosphatase activity. The reason for this is unclear. Calcitonin alone had no effect on acid phosphatase activity, but when added together with PTH, inhibited the stimulatory effect of PTH.

The multinucleated cells appear to arise by fusion of mononuclear precursors rather than endomitosis. Time-lapse cinemicrographic studies showed mononuclear cells fusing with multinucleated cells. The fusion process occurred within 12 to 16 h. Further support that the multinucleated cells arise by fusion was seen in the autoradiographic studies. The nuclei in the majority of multinucleated cells were not labeled when the cultures were incubated with [3H]thymidine for 24 h, washed, and then cultured with PTH for an additional 18 h (Fig. 6). We found that the multinucleated cells that contained labeled nuclei also contained predominantly one labeled nucleus, with the other nuclei in the cell being unlabeled.

Fractionation of feline marrow cells on discontinuous Percoll density gradients enriched multinucleated cell progenitors (Table V). The progenitor cells were found predominantly in layers of density 1.07-1.08 gm/ml and with Wright's staining were immature monocyte-macrophages histologically. The

| Days in culture | Expt. 1 | Expt. 2 |
|----------------|--------|--------|
| 0              | 0      | 0      |
| 1              | 0      | 0      |
| 2              | 2      | 2      |
| 3              | 16     | 5      |
| 4              | 19     | 15     |
| 5              | 24     | 18     |
| 6              | 14     | 17     |
| 7              | 16     | 22     |
| 9              | 16     | 11     |
| 16             | 38     | 38     |
| 24             |        |        |

Unfractionated cat bone marrow was cultured in alpha-MEM with 10% horse serum as described in Materials and Methods. Results represent the mean of two independent determinations. Cells were counted at X 250 (20 random fields), and the number of cells containing three or more nuclei was scored.

![Figure 1](Image)

Light microscopic appearance of multinucleated cells. Cells were processed and stained as described in Materials and Methods. The cells have a granular cytoplasm, with a clear peripheral cytoplasm and prominent nucleoli. Bar, 50 μm. X 300.
FIGURE 2  Typical ultrastructural morphology of a cultured multinucleated cell derived from cat bone marrow mononucleated cells. Cat marrow cells were cultured for 14 d and then processed for routine electron microscopy (see Materials and Methods). This multinucleated cell contains abundant cytoplasmic organelles including lipid droplets, rough endoplasmic reticulum and branched mitochondria. The peripheral cytoplasm (arrow) lacks organelles other than free ribosomes. Bar, 5 μm. × 4,900.
FIGURE 3 Cell surface specialization in cultured feline osteoclasts. (A) Extensive cytoplasmic involutions are present at the periphery of this multinucleated cell. Vacuoles present in this area contain granular material and have focally thickened areas of membrane that resembles coated pits (arrow). Bar, 1 μm. × 11,700. (B) Thin and delicate cytoplasmic extensions are present at the surface of this cell, which also contains numerous vacuoles. Bar, 1 μm. × 12,800. (C) In addition to surface folding, the plasmalemmal membrane of this multinucleated cell has focal, thickened areas resembling coated pits (arrowhead), several of which seem to be internalized (arrow). Bar, 1 μm. × 27,000
mononuclear cells stained heavily with nonspecific esterase, a monocyte stain, but did not stain with granulocytic esterase, or periodic-acid Schiff stain, a lymphocyte stain. The progenitor cells were predominantly nonadherent to plastic. Cells initially adherent to plastic formed 26 multinucleated cells per 20 microscopic fields while nonadherent marrow cells formed 149 multinucleated cells per 20 microscopic fields. Removal of cells adherent to plastic from unfractionated marrow cells enriched progenitors threefold as compared to unfractionated marrow. The multinucleated cells that formed also were stained with nonspecific esterase but did not stain with periodic-acid Schiff stain or granulocytic esterase, a staining pattern seen in monocyte-macrophages. The nonspecific esterase staining was most pronounced in the smaller multinucleated cells containing three to six nuclei, and was decreased or absent in the larger more multinucleated cells. These multinucleated cells were phagocytic (Fig. 7, arrow), a characteristic of monocyte-macrophages.

The multinucleated cells resorbed bone in vitro as shown by the release of $^{45}$Ca from devitalized bone particles. Cultures enriched for multinucleated cells showed significant bone resorption. 42 ± 3% $^{45}$Ca was released by these cultures (total counts in bone particles added = 1,290 ± 77 cpm) compared to 20 ± 3% (total counts added = 1,725 ± 170 cpm) for cultures not containing cells. Addition of PTH did not affect bone resorption and calcitonin inhibited resorption in two

| Total cells scored | Multinucleated cells | Percent of cells scored | No. of nuclei per cell |
|--------------------|----------------------|-------------------------|-----------------------|
| Control            | 1,541 ± 181          | 9 ± 1                   | 0.60                  | 4.43 ± 0.35             |
| PTH                | 1,509 ± 181          | 19 ± 3*                 | 1.3                   | 4.55 ± 0.56             |
| Calcitonin         | 1,528 ± 123          | 10 ± 3                  | 0.65                  | 4.24 ± 0.35             |
| Calcitonin and PTH | 1,596 ± 183          | 7 ± 2                   | 0.43                  | 4.67 ± 0.49             |

Unfractionated feline bone marrow cells were cultured in MEM containing 30% horse serum as described in Materials and Methods. After 2 wk of culture PTH (200 ng/ml), calcitonin (100 ng/ml), or PTH (200 ng/ml) and calcitonin (100 ng/ml) were added. The cultures were incubated an additional 24 h, and then fixed with glutaraldehyde and stained with Wright's stain. The number of cells containing 3 or more nuclei were scored in 20 microscopic fields (x 400). Results represent the mean ±1 SEM of triplicate determinations from three separate experiments. Differences were determined by one way analyses of variance for repeated measures and a Newman-Keuls multiple comparison procedure.

* Significantly different from control group at $P < 0.01.$
TABLE III
Effect of Prostaglandin E2 and 1,25(OH)2 Vitamin D3 on Formation of Multinucleated Cells from Unfractionated Feline Marrow Cultures

|                          | Total no. of cells | Multinucleated cells | Percent of cells scored | No. of nuclei cell |
|--------------------------|--------------------|----------------------|-------------------------|--------------------|
| Control                  | 1,769 ± 168        | 13 ± 6               | 0.7                     | 5.11 ± 0.57        |
| Prostaglandin E2         |                    |                      |                         |                    |
| 10^{-3} M                | 2,508 ± 633        | 160 ± 6*             | 6.4                     | 4.1 ± 0.69         |
| 10^{-4} M                | 3,072 ± 249        | 120 ± 6*             | 3.9                     | 5.04 ± 0.49        |
| 10^{-5} M                | 2,126 ± 204        | 100 ± 12*            | 4.9                     | 5.37 ± 0.52        |
| 1,25(OH)2 vitamin D3     |                    |                      |                         |                    |
| 10^{-4} M                | 2,225 ± 245        | 280 ± 80*            | 12.6                    | 5.09 ± 0.55        |
| 10^{-5} M                | 2,182 ± 356        | 240 ± 87*            | 11.0                    | 4.26 ± 0.14        |
| 10^{-6} M                | 1,952 ± 149        | 100 ± 53*            | 5.1                     | 5.36 ± 0.58        |

Unfractionated feline marrow cells were cultured for 2 wk and then prostaglandin E2 or 1,25(OH)2 vitamin D3 were added in various concentrations. The cells were incubated an additional 24 h and then were fixed and stained. The number of cells containing 3 or more nuclei were scored in 20 microscopic fields (× 250). Results are given as mean ± 1 SEM for triplicate determinations from three separate experiments.

* Significantly different from control (P < 0.01).

TABLE IV
Effect of PTH (200 ng/ml), Calcitonin (100 ng/ml), and Tartrate (27 mM) on Acid Phosphatase Activity of Multinucleated Cells from Feline Marrow Cultures

|                          | Total acid phosphatase | Percent increase |
|--------------------------|------------------------|-----------------|
| Control                  | 1.17 ± 0.04            | -               |
| PTH                      | 2.13 ± 0.11            | +82 ± 7*        |
| Calcitonin               | 0.84 ± 0.23            | -28 ± 16*       |
| Calcitonin and PTH       | 0.71 ± 0.06            | -40 ± 5*        |
| PTH + tartrate           | 2.41 ± 0.05            | +105 ± 5        |

Acid phosphatase activity was detected histochemically and cells were scored by the intensity of granular staining. Cells were graded from 3 (intense granulation) to 0 (no discernible activity). All cells were scored at random without knowledge of the treatment group. Scores were totaled and divided by total number of multinucleated cells scored to give mean score per cell. Cells were counted in 20 random fields in each well (at least 60 cells per group) at × 1,000. Results are mean and ± 1 SEM of triplicate determination from three separate experiments. Differences were assessed by one way analysis of variance for repeated measurements and a Newman-Keuls multiple comparison procedure.

* P < 0.01, compared to control group.

#DISCUSSION

We have demonstrated that the multinucleated cells in long-term feline marrow cultures have many of the characteristics of osteoclasts. The cells in our cultures were large and multinucleated with ultrastructural features of osteoclasts, including a peripheral cytoplasmic clear zone devoid of subcellular organelles, and extensive cell surface folds and branched pleomorphic mitochondria. These particular ultrastructural features are found in freshly isolated osteoclasts and in osteoclasts not lying adjacent to an endosteal bone surface (7). Although numerous plasmalemmal folds and indentations were also regularly observed in our cultured cells, we did not identify the classical ruffled borders as reported for osteoclasts found adjacent to bone in Howship's lacunae in vivo (17). The absence of a classical ruffled border in our cultured cells may be due to our inability to mimic the true endosteal bone surface in vitro. Nonetheless, the multinucleated cells did demonstrate a marked increase in cytoplasmic membrane folds in the presence of bone (Fig. 9).

The multinucleated cells in our cultures had other charac-
TABLE V
Enrichment of Feline Marrow Multinucleated Cell Progenitors, by Percoll Density Gradient Centrifugation

| Density (g/ml) | Cell per layer (% Total) | Initial predominant morphology | Multinucleated cells | Fractionated/unfractionated |
|---------------|--------------------------|-------------------------------|----------------------|-----------------------------|
| 1.050         | 11.1                     | 1.4                           | Myeloblasts          | 0                           | 0                           |
| 1.055         | 10.5                     | 1.3                           | Myelocytes           | 0                           | 0                           |
| 1.060         | 15.4                     | 2.1                           | Myelocytes           | 6                           | 1                           |
| 1.065         | 632.0                    | 86.7                          | Metamyelocytes       | 166                         | 4                           |
| 1.070         | 36.4                     |                               | Myeloid bands, monocytes | 1,000                       | 23                          |
| 1.075         | 12.7                     | 1.7                           | Myeloid bands, monocytes | 1,400                       | 32                          |
| 1.080         | 8.0                      | 1.0                           | Normoblasts          | 160                         | 4                           |
| 1.085         | 3.1                      | 0.4                           | Normoblasts          | NC                          | 0                           |
| 1.090         | —                        | —                             | Erythrocytes         | NC                          | 0                           |
| Unfractionated| —                        | —                             | —                    | 44                          | 1                           |

Feline marrow cells were layered over Percoll density gradients and the cells from each fraction cultured for 14 d as described in Materials and Methods. The marrow cultures from each layer were fixed, stained, and the number of cells containing three or more nuclei in 20 microscopic fields (x 250) were scored. Cytospin cytology was done on each layer prior to culture. The results are from one experiment. In four separate studies multinucleated cell progenitors were enriched 4- to 32-fold. Lymphocytes were seen in all layers of the gradient. NC, not cultured because of insufficient cell numbers.

FIGURE 7 A multinucleated cell in feline marrow cultures stained for nonspecific esterase. Bone marrow cells were cultured for 14 d before processing for nonspecific esterase staining as described in Methods. Arrow denotes a phagocytosed cell. Bar, 50 μm. x 300.

FIGURE 8 Light microscopic appearance of cat osteoclasts co-cultured with dritvalized bone particles. Bone marrow cells were cultured for 14 d; bone particles were then added and the cultures incubated for 48 h prior to fixation decalcification and processing for electron microscopy. Thick sections (1 μm) were cut and stained with toluidine blue. A multinucleated cell is shown attached to a bone particle (B). Note the numerous lipid droplets (small, dark cytoplasmic granules) in these cells and their relative paucity in areas adjacent to the bone particle (arrow). Bar, 25 μm. x 800.

FIGURE 9 Extensive membrane ruffling of a multinucleated cell grown in the presence of bone particles for 72 h. Bar, 1 μm. x 6,800.

was inhibited by calcitonin. Osteoclasts have been shown before to form by fusion (18), and the results of the current investigation suggest that this process is probably stimulated by PTH and inhibited by calcitonin (Table II). The multinucleated cells in our cultures also contained an acid phosphatase which was tartrate-resistant and which was increased in the presence of PTH and was inhibited by calcitonin (Table IV). This property is also characteristic of the osteoclast (14, 19, 20).

Our data suggests that the mononuclear precursor cell of these multinucleated cells is immature marrow monocyte-macrophage. The progenitor cells stained heavily with nonspecific esterase, were phagocytic and appear similar to other monocytes-macrophages with Wright's-Giemsa staining. These data agree with studies of Burger et al. (21), who also
found that the precursor to their multinucleated cells were marrow monocytes that initially were not adherent to plastic. Similarly, in vivo studies of Gothlin and Ericsson (5) also suggested that marrow monocytes give rise to osteoclasts.

The progenitor of the osteoclast-like cell in our cultures appears responsive to osteotropic hormones since PTH, vitamin D, and prostaglandin E2 increased multinucleated numbers while calcitonin blocked the effects of PTH (Tables II and III). Bingham et al. (22) and Tatevosian (23) have shown that administration of parathyroid extracts to rabbits or mice increases the number of osteoclasts in vivo. Similarly administration of vitamin D and prostaglandins increase osteoclast numbers in vivo (24). These data suggest that osteoclast progenitors are responsive to osteotropic hormones, and are consistent with our in vitro observations.

The multinucleated cells appear to form by fusion of mononuclear precursors based on time-lapse cinemicrography and autoradiographic studies. However, such studies cannot differentiate between phagocytosis of labeled mononuclear cells and fusion of mononuclear cells to form multinucleated cells. Fusion is the more likely mechanism for osteoclast formation, since the nuclei of the multinucleated cells are not pyknotic and appear normal ultrastructurally, whereas a phagocytosed cell should be degraded. Multinucleated cells form within 18 h after addition of PTH to the cultures, suggesting that it is unlikely that endomitosis is the mechanism responsible for the formation of the osteoclasts. If endomitosis is the mechanism of multinucleated cell formation, the cell cycle time of the progenitors must be extremely short. Further supporting that endomitosis is not responsible is the observation that the nuclei in the majority of the multinucleated cells were not labeled by [3H]thymidine, and therefore are not actively synthesizing DNA. The multinucleated cells which did contain labeled nuclei predominantly (>90%) had only one labeled nucleus with the other nuclei unlabeled. Previous in vivo as well as in vitro studies have suggested that osteoclasts arise from fusion of mononuclear precursors (1, 2, 18).

It appears that the progenitors of the multinucleated cells in our cultures behave differently from mature monocyte-macrophages. The progenitor cell is initially nonadherent to plastic and responds to osteotropic hormones. Mundy et al. (25) demonstrated that peripheral blood monocytes could resorb bone but were unresponsive to osteotropic hormones. Osdoby et al. (7) cultured chick peripheral blood monocytes and demonstrated that they gave rise to multinucleated cells that differed ultrastructurally from osteoclasts. Taken together, these data suggest that the osteoclast progenitor is not identical to monocytes found in peripheral blood, but may be a less differentiated marrow monocyte precursor that can give rise to osteoclasts in the appropriate microenvironment. The nonspecific esterase staining of the osteoclast-like cells and their phagocytic ability further supports their monocytic origin. Ultrastructurally, these progenitor cells may be the monocytoïd cells with branched mitochondria present in our cultures. Further studies will be required to confirm whether these cells are the progenitors of these osteoclast-like cells.

Enriched populations of cells released 45Ca from devitalized bone particles, and the membranes of cells grown in the presence of bone particles showed extensive folding and ruffling. However, we could not consistently demonstrate hormonal control of 45Ca release. This lack of hormonal responsiveness may be due to the use of devitalized bone particles in our studies. Devitalized bone may not have been suitable for demonstrating such effects because of the absence of other bone cell types or changes in matrix structure in devitalized bone. The mechanism by which PTH stimulates bone resorption by osteoclasts is unknown. Possibly PTH acts on other cells in bone such as osteoblasts that in turn affect osteoclast function (26). The variable effect of calcitonin in inhibiting resorption may be due to loss of calcitonin sensitivity, by the osteoclast-like bone cells in our cultures. Wener et al. (27) have shown that bone cultures treated with calcitonin lose sensitivity to the hormone and resorb in the continued presence of calcitonin.

Microenvironmental factors appear important in osteoclast formation. The culture system we employed results in formation of multinucleated giant cells responsive to osteotropic hormones capable of bone resorption and possessing many of the physical and ultrastructural characteristics of osteoclasts. This culture system is dependent on the formation of a marrow adherent cell layer which mimics the marrow microenvironment (28–30). However, using a similar technique Dexter and co-workers (28–30) did not report the formation of multinucleated cells in their bone marrow cultures. Their marrow cultures routinely contained hydrocortisone, while ours did not. When hydrocortisone was added initially to our cultures, no multinucleated cells formed (data not shown). Therefore, hydrocortisone may inhibit osteoclast formation in vitro, and may explain why these authors have not reported multinucleated cells in vitro. Suda et al. (31) have also observed that hydrocortisone inhibits feline osteoclast formation in vitro.

There are other approaches being developed for studying osteoclasts in vitro. Pre-existing osteoclasts stripped from the endosteal surfaces of growing bones have been used by Zallone et al. (32) and Osdoby et al. (7). However, these cells do not allow studies of the differentiation of the mature cell from its precursors, nor do they permit the identification of precursors. Furthermore, the viability of the cell preparations is often low. Cells have also been obtained from rodent calvaria by enzyme digestion (33) or prior culture with PTH (34). However, these techniques will not provide an approach for studying osteoclasts derived from normal humans or patients with diseases associated with bone resorption as the technique described in this report does.

The ability to grow osteoclasts in vitro and enrich their progenitors will permit basic studies on bone cell biology. Such culture systems may help in elucidating other factors that may affect osteoclast formation and activity in normal and pathologic states.

We wish to thank Pat Cooper and Nancy Garrett for preparing the manuscript.

This study was supported in part by merit review funds from the Veterans Administration and by grants AM28149 and AM28639 from the National Institutes of Health.

Received for publication 8 November 1983, and in revised form 30 April 1984.

REFERENCES

1. Jotereau, F. V., and N. M. LeDouarin. 1978. The developmental relationship between osteocytes and osteoclasts: a study using the quail-chick nuclear marker in endochondral ossification. Dev. Biol. 63:253–263.
2. Fischman, D. A., and D. E. Hay. 1962. Origin of osteoclasts from monocytic leukocytes in regenerating newt limbs. Anat. Rec. 143:239–338.
3. Marks, S. C. 1978. Studies of the cellular cure for osteopetrosis by transplanted cells: specificity of the cell type in IA rats. Am. J. Anat. 151:131–137.
4. Buring, K. 1975. On the origin of cells in heterotopic bone formation. Clin. Orthopaed. 110:293–302.
5. Gothlin, G., and J. L. E. Ericson. 1973. On the histogenesis of the cells in fracture callus: electron microscopic autoradiographic observations in parabiotic rats and studies on labeled monocytes. Virchows Arch. B Cell Pathol. 12:318–329.
6. Walker, D. G. 1975. Control of bone resorption by hematopoietic tissue. The induction and reversal of congenital osteopetrosis in mice through use of bone marrow and splenic transplants. J. Exp. Med. 142:651–663.
7. Odosby, P., M. C. Martini, and A. I. Caplan. 1982. Isolated osteoclasts and their presumed progenitor cells, the monocyte, in culture. J. Exp. Zool. 224:331–344.
8. Zuckerman, Sh. H., S. K. Ackerman, and S. D. Douglas. 1979. Long-term human peripheral blood monocyte-culture: establishment, metabolism and morphology of primary human monocyte-macrophage cell cultures. Immunology, 38:401–411.
9. Wong, G. L., and D. V. Cohn. 1974. Separation of parathyroid hormone and calcitonin-sensitive cells from nonresponsive bone cells. Nature (Lond.). 252:713–714.
10. Luben, R. A., G. L. Wong, and D. V. Cohn. 1976. Biochemical characterization with parathyroid hormone and calcitonin of isolated bone cells: provisional identification of osteoclasts and osteoblasts. Endocrinology, 96:526–533.
11. Testa, N. G., T. D. Allen, L. G. Latsha, D. Orion, and O. Jarret. 1981. Generation of osteoclasts in vitro. J. Cell Sci. 47:127–137.
12. Nelson, D. A., and F. R. Davey. 1977. Leukocyte Esterases in Hematology. W. J. Williams, E. Bentler, A. J. Ersler, and R. W. Rundles, editors. McGraw-Hill, New York.
13. Karnovsky, M. J. 1967. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol. 37:137A. (Abstr.)
14. Hammarstrom, L. E., J. S. Hanker, and S. U. Toverud. 1971. Cellular differences in acid phosphatase isoenzymes in bone and teeth. Clin. Orthop. Rel. Res. 78:151–167.
15. Rodman, G. D., J. J. Hutton, and F. J. Bollum. 1976. DNA polymerase, thymidine kinase and DNA synthesis in erythropoietic mouse spleen cells separated or bovine serum albumin gradients. Biochem. Biophys. Acta. 425:478–491.
16. Teitelbaum, S. L., C. C. Stewart, and A. J. Kahn. 1979. Rodent peritoneal macrophages as bone resorbing cells. Calcif. Tissue Int. 27:255–261.
17. Holtrop, M. E., and E. J. King. 1977. The ultrastructure of the osteoclast and if functional implications. Clin. Orthop. Rel. Res. 123:176–196.
18. Lee, W. S. S., and P. D. Nolan. 1963. Origin of osteoclasts from the fusion of phagocytes. Nature (Lond.). 200:225–226.
19. Doty, S. B., and B. M. Schofield. 1972. Electron microscopic localization of hydrolytic enzymes in osteoclasts. Histochem. J. 4:245–258.
20. Vaes, G. M. 1968. On the mechanism of bone resorption. The action of parathyroid hormone on the excretion and synthesis on the lysosomal enzymes and on the extracellular release of acid by bone cells. J. Cell Biol. 39:676–696.
21. Burger, E. H., J. W. M. Van Der Meer, J. S. Van De Gevel, J. C. Griehnau, C. W. Thesingh, and R. Van Furth. 1982. In vitro formation of osteoclasts from long term bone cultures of bone marrow mononuclear phagocytes. J. Exp. Med. 156:1604–1614.
22. Bingham, P. J., I. A. Brazell, and M. Owen. 1969. The effect of parathyroid extract on cellular activity and plasma calcium levels in vivo. J. Endocrinol. 45:387–400.
23. Tatrosianian, A. 1973. Effect of parathyroid extract on blood calcium and osteoclast count in vivo. Calcif. Tissue Res. 11:251–257.
24. Hefley, T. J., and F. R. Cohn. 1982. Isolated osteoclasts from fetal rat long bones. Calcif. Tissue Int. 34:480–487.