Journal of Cellular Biochemistry 72:67–80 (1999)

Osteoclast Markers Accumulate on Cells Developing From Human Peripheral Blood Mononuclear Precursors

Judy Faust,[1] Dave L. Lacey,[1] Pamela Hunt,[1] Teresa L. Burgess,[2] Sheila Scully,[1] Gwyneth Van,[1] Alana Eli,[1] Yi-xin Qian,[2] and Victoria Shalhoub[1]*

[1]Department of Pathology, Amgen Inc., Thousand Oaks, California 91320
[2]Department of Mammalian Cell Molecular Biology, Amgen Inc., Thousand Oaks, California 91320

Abstract Recent studies show that human osteoclasts develop in vitro from hematopoietic cells; however, special cultures conditions and/or cytokine mobilized peripheral blood are apparently required. Here, we report that cells expressing osteoclast markers differentiate from precursors present in nonmobilized peripheral blood mononuclear cells (PBMC), without the addition of stromal cells, growth factors, cytokines or steroids; and characterize their phenotype. Three days after establishing high-density PBMC cultures (1.5×10^6 cells/cm^2), in serum-containing medium, small adherent colonies of tartrate resistant acid phosphatase positive (TRAP+) cells emerge, amidst massive monocyte cell death. These adherent cells have an eccentrically placed, round nucleus, and express low levels of TRAP and sodium fluoride-resistant- α-naphthyl-acetate-esterase (NaF-R-NSE). Over the next week, this cell population accumulates phenotypic markers of osteoclasts (vitronectin receptor [VR], calcitonin receptor, TRAP, cathepsin K protein, and mRNA) with increased nuclearity, covering the entire surface by 15 days. When cultured on bone, VR+, TRAP+ cells of low multinuclearity appear and cover up to 50% of the surface. Resorption lacunae can be observed by day 22. Although these pits are not nearly as numerous as the cells of preosteoclast phenotype, they do represent the activity of a subset of osteoclast-like cells that has achieved osteoclastic maturity under these culture conditions. Transcripts for osteoprotegerin ligand (OPGL), an osteoclast differentiation factor (also known as RANKL and TRANCE) are expressed, likely by adherent cells. Thus, an adherent population of cells, with preosteoclast/osteoclast phenotypic properties, arises selectively under simple culture conditions from normal PBMC. Further characterization of these cells should identify factors involved in the growth, terminal differentiation and activation of osteoclasts. J. Cell. Biochem. 72:67–80, 1999.

Key words: bone resorption; cathepsin K; vitronectin receptor; tartrate resistant acid phosphatase; preosteoclast development

Osteoclasts are the mononuclear or multinucleated cells that resorb bone [Kaye, 1984; Marks and Popoff, 1988; Fuller and Chambers, 1987; Sarma and Flanagan, 1996]. The nature of osteoclast precursor cells has intrigued researchers for years. Early studies involving chick-quail chimeras [Kahn and Simmons, 1975], parabiotic animals [Walker, 1973], and bone marrow transplantation in osteopetrotic animals [Walker, 1975] and children [Coccia et al., 1980] have shown that osteoclast precursors originate in hematopoietic tissues. More recent evidence indicates that osteoclasts develop from the pluripotent hematopoietic stem cell [Sheven et al., 1986; Hattersley and Chambers, 1989; Hagenaars et al., 1989; Matayoshi et al., 1996].

Recently, the generation of human osteoclasts from a variety of sources, including granulocyte-colony stimulating factor (G-CSF) mobilized peripheral blood [Purton et al., 1996; Matayoshi et al., 1996], diseased [Gregoretti et al., 1995] and normal peripheral blood/mouse stromal cell cocultures [Fujikawa et al., 1996], bone marrow [Sarma and Flanagan, 1996; Roodman, 1995; Kassem et al., 1991], osteoclastoma tissue [Grano et al., 1994; James et al., 1996], and cord blood [Roux et al., 1996], has been documented. While these reports have advanced our understanding of human osteoclastogenesis, much remains to be learned about the precise roles of stromal cells, steroids (such as Vitamin D and glucocorticoids, estrogen), and cytokines (such as colony stimulating fac-

*Correspondence to: V. Shalhoub, Amgen, Inc., 15-2-B, Amgen Center, Thousand Oaks, CA.

Received 10 June 1998; Accepted 10 July 1998

tor-1 [CSF-1]). Although osteoclasts have been obtained from G-CSF mobilized blood in the absence of stromal cells [Purton et al., 1996; Roux et al., 1994], there are no reports of osteoclast generation from normal peripheral blood, in the absence of stromal cells [Purton et al., 1996; Matayoshi et al., 1996]. Functional osteoclasts are reported to form from normal PBMC only in the presence of stromal cells such as mouse preadipocytic ST2 or rat-osteoblast like UMR106 cells, and steroids [Fujikawa et al., 1996]. Furthermore, a systematic characterization of the formation and development of osteoclasts, derived from progenitor cells has not been performed, although notable progress in this area has been made in rat [Baron et al., 1986], and human osteophytic bone [Connor et al., 1995] and osteoclastoma systems [James et al., 1996].

The goals of this study were to develop an in vitro system that generates human preosteoclasts and/or osteoclasts from normal peripheral blood in the absence of stromal cells; and, to characterize the sequence of the cellular phenotypes that are associated with osteoclast development.

We found that cells with preosteoclast phenotypic properties can form from human peripheral blood mononuclear cell (PBMC) precursors when cultured at high density (1.5×10^6 cells/cm^2) in alpha minimum essential medium (αMEM) + 10% heat inactivated fetal bovine serum (FBS). Homogeneous cultures of adherent cells form in colonies in the presence of non-adherent lymphocytes by day 5 when most other cells (monocytes) in culture have died. By 2 weeks, when cultured on plastic, the adherent cells are mono-, di-, or multinucleated, and display protein and mRNA for the vitronectin receptor (VR+), cathepsin K (CathK+), tartrate resistant acid phosphatase (TRAP+), and the calcitonin receptor (CR+); and, possess a sodium fluoride-resistant α-naphthyl acetate esterase (NaF-R-NSE+). When cultured on bone, in the absence of stromal cells, abundant cells, expressing the preosteoclast/osteoclast phenotype are generated and some are capable of bone resorption. This system should help identify the factors and mechanisms involved in human osteoclast progenitor cell development, as well as, potentially, those required for mature osteoclast function.

MATERIALS AND METHODS

Tissue Culture

Human mononuclear cells were prepared from both leukapheresis product (HemaCare Corporation, Sherman Oaks, CA) and heparinized peripheral blood (PB), with donor consent. The leukapheresis product was diluted to 500 ml with Hanks Balanced Salt Solution (HBSS, Gibco-BRL, Gaithersburg, MD), centrifuged (460*g*, 10 min), rinsed and aliquots (12.5 ml) layered over Ficoll/Hypaque (F/H) (25 ml, Pharmacia, Piscataway, NJ). Following centrifugation (460*g*, 25 min), the interface cells were collected, diluted with HBSS and recentrifuged (460*g*, 20 min). The cells were then suspended in HBSS, counted, recentrifuged (460*g* 8 min), and, resuspended in Iscove's Modified Dulbecco's Medium (IMDM; approximately 10^9 cells/200 ml), supplemented with penicillin, streptomycin, and glutamine. The cells were stored overnight at 4°C prior to use. For PB, heparinized blood (5 ml) was centrifuged (460*g*, 30 min) over F/H (3 ml). The interface cells were collected and washed twice with alpha minimum essential medium (αMEM) + 10% fetal bovine serum (FBS). The cell yields were approximately 1×10^9 and 1×10^7 mononuclear cells from one leukapheresis product and 10 ml of peripheral blood, respectively. Cells were cultured in αMEM + 10% FBS (Cansera, Rexdale, Ontario, Canada), supplemented with 100 IU/ml penicillin, 100 ug/ml streptomycin sulfate, 250 ng/ml fungisone (Bio WHITTAKER, Walkersville, MD). Cells were plated at 1.5×10^6 cells/cm^2 in 24-well plates or eight-well chamber slides (Nunc, Inc., Naperville, IL). Cells were fed every 2–3 days, by replacing half the media up to day 7. Thereafter, all the media was removed and replenished until the end of the culture period.

Osteoclast Resorption Assay

Circular cortical bovine bone slices (6 mm diameter x 0.8 mm) were prepared using a low speed diamond saw (Buehler, Evanston, IL), and were cultured with the PBMC in 24 well plates for up to 3 weeks. Media was removed, cells washed with phosphate buffered saline (PBS), fixed with 2% paraformaldehyde or acetone and assessed for tartrate resistant acid phosphatase (TRAP) activity or VRs using immunohistochemistry (see below). Cells were then removed mechanically after incubating

the bone slices in 10% sodium hypochlorite for 10 minutes; or, by sonication (30 second pulses), sequentially, twice in 2% ammonium hydroxide, twice in distilled water and twice in acetone. Resorption lacunae were initially visualized using toluidine blue (0.1%). Photographs were obtained using a NIKON MICROPHOT-FXA microscope and SONY video printer (Trinitron). The bones were then dehydrated in alcohol, and sputter coated with 30 nm gold using a Hummer VII sputtering system (Anatech Ltd, Alexandria, VA). Resorption lacunae were visualized using a JEOL JSM-5200 Scanning Microscope (Jeol Technics Ltd., Tokyo, Japan).

Histochemistry

Every 2–3 days, cells were gently removed from the plates using a plastic pasteur pipette together with cells in the culture medium. Cytospin preparations and bones were fixed with 2% paraformaldehyde or acetone for 10 minutes and stored at room temperature, or 4°C, respectively. Cells on cytocentrifuge preparations were stained with Wright's/Giemsa. For valid comparison of enzyme activity, staining for α-naphthyl acetate esterase (NSE), and TRAP (both from Sigma, St. Louis, MO) was performed for all timepoints, after the experiment was completed in a single run using freshly prepared reagents according to the manufacturer's instructions. The NSE reaction was performed ± 18 mM NaF because monocyte NSE is sensitive to 18 mM sodium fluoride (NaF-S-NSE) while NSE present in nonmonocytic cells or specialized monocytes is resistant to NaF (NaF-R-NSE) (Sun, 1985). Photographs were obtained with a NIKON MICROPHOT-FXA microscope and NIKON DX-DB2 camera, using 20× and 60× objectives.

Antibodies

Monoclonal antibodies to the VR (23c6) and control antibodies (murine IgG1) were obtained from Pharmingen (San Diego, CA). The anti-Cathepsin K (CathK) antibody was generated in rabbits using a KLH-coupled synthetic peptide corresponding to a portion of human CathK (MYNPTGKAAKCRGYREIPEGNEK). This sequence differs significantly from the corresponding regions of Cathepsin S and L [Bossard et al., 1996]. The antiserum was affinity purified against the peptide as described [Burgess et al., 1995].

Immunocytochemistry

Acetone-fixed slides, stored at 4°C, were equilibrated at room temperature until dry, and incubated with normal horse serum for 15 minutes, at room temperature. The slides were then incubated for 2 hours at room temperature with 23c6 or IgG1 at final concentrations of 10 µg/ml. After rinsing with PBS, the slides or bones were treated with 3% H_2O_2 in methanol (1:3) for 30 minutes, rinsed, incubated with biotinylated horse anti-mouse antibody for 25 minutes, rinsed, and incubated with avidin-biotin-peroxidase complex for 25 minutes. After rinsing, DAB-substrate-chromogen solution was applied for five minutes. Prior to coverslipping, the slides were rinsed and counterstained with hematoxylin for one minute, washed with tap water, dehydrated in 70% ethanol and absolute ethanol, washed with xylene and mounted with a cover slip with mounting medium. The bones were dehydrated, air dried, and then placed on a drop of mounting medium. Slides and bones were photographed with a NIKON MICROPHOT-FXA microscope with a SONY videoprinter (Trinitron), and NIKON DX-DB2 camera, respectively.

Western Blots

For each timepoint, cells covering approximately 6 cm^2 of growth area were collected in the culture media by gently scraping with a plastic pipette. The cells were centrifuged, and lysed with 100 microliters of nonidet P-40 (Sigma Chemical Company). After centrifugation, the nuclei were discarded, and the supernatant frozen at -20°C. Proteins present in 40 microliters of this cytoplasmic extract, per timepoint, were separated using SDS PAGE and 10% gels. Following transfer, the blots were probed with the affinity purified anti-cathK antibodies (12 µg/ml) in the presence and absence of competing peptide (3 µg/ml). The primary antibody was detected using HRP-conjugated anti-rabbit and an ECL kit from Amersham (Arlington Heights, Ill).

Calcitonin receptor detection. Slides were incubated with 0.4 nM human ^{125}I calcitonin (specific activity, 2000 Ci/mM, Amersham, Arlington Heights, Ill.) in αMEM+0.1% BSA for 1 hour at room temperature. Specific binding was assessed by addition of excess unlabeled human calcitonin (at a final concentration of 3×10^{-7} nM) (Sigma Chemical Company).

The cells were then extensively washed with PBS, fixed with 2% paraformaldehyde, stained for TRAP, covered with Kodak NTB-2 emulsion, and developed after a 2 week exposure at -80° C.

In situ hybridization. Cytocentrifuge slides, containing cells from 12 day cultures, were rinsed with Ca^{++} and Mg^{++} containing PBS, fixed with 0.5% paraformaldehyde in PBS, at room temperature, for 2 minutes, washed twice in PBS, drained of excess buffer, and fixed for 10 minutes in 5% acetic acid in absolute ethanol at -20°C. The acid was removed by rinsing twice with 95% ethanol, and twice with 100% ethanol. Slides were air dried and stored at -70°C. *In situ* hybridization was performed as described (Wilcox, 1993), using ^{33}P-labelled riboprobes corresponding to nucleotides 1481–1797, sense and antisense mouse CR sequence (Gb:U18542), and cells counterstained with hematoxylin and eosin. Slides were counterstained with hematoxylin and eosin. *In situ* hybridization to calcitonin receptor (CR) mRNA in osteoclasts generated in 7 day day cocultures of mouse marrow and ST2 cells [Lacey et al., 1995], with antisense and sense mouse probes, was also performed as a control.

RNase protection assays. Mononuclear cells obtained from leukapheresis product were cultured as described above at 1.2 x 10^8 cells per 100 mm dish. RNA was collected at the time of plating (day 1), and on days 5, 9, 12, 15 and 18, by first washing the cells with PBS to remove lymphocytes. The adherent cells were then scraped off the dishes in PBS, and pelleted in 50 ml plastic tubes by centrifugation at 460 x g for 5 minutes. In a second experiment, cells were lysed directly on the plate. RNA was then isolated using the Qiagen RNeasy Mini Kit (Qiagen Inc., Santa Clarita, CA). The integrity of RNA was ascertained by fractionation on a 6.6% paraformaldehyde-1% agarose gel, and staining with ethidium bromide to visualize intact 28S and 18S ribosomal bands.

RNase protection assays were performed using the RPA II kit (Ambion, Inc., Austin, TX) and 2 ug of total cellular RNA with ^{32}P labelled RNA probes for human CathK (Gb#U13665, nucleotide numbers (nts) 682–1041); human β3 (subunit of vitronectin receptor) (Gb#J02703, nts 952–1214); human c-src (Gb#M16237, nts 293–467); human PU.1 (Gb#X52056, nts 494–700,); human TRAP (Gb#X14618, nts 420–608,); human OPGL (Gb#AF053712, nts 371–659) and cyclophilin (Ambion). Following an overnight exposure, signal was measured using a phosphorimager (molecular Dynamics, Sunnyvale, CA) and ImageQuant software. Levels of CathK, β3, c-src, PU.1 TRAP and OPGL were normalized to cyclophilin mRNA levels.

RESULTS

Previous reports demonstrated osteoclast formation from G-CSF-mobilized peripheral blood (PB) [Purton et al., 1996], and from CD34+ cells purified from G-CSF-mobilized PB [Matayoshi et al., 1996] in the absence of stromal cells. Our preliminary experiments demonstrated that cells with preosteoclast/osteoclast morphology form from *normal* PB, in the absence of stromal cells, when peripheral blood mononuclear cells (PBMC) are plated on plastic or bone, at high density (1.5 x 10^6 cells/cm^2). Thus, we attempted to characterize these cells as they develop over a period of 3 weeks in culture.

On Day 11, bone-adherent cells express membrane associated VR, demonstrated by 23c6 antibody staining (Fig. 1A), and TRAP (Fig. 1B). Cells on bone, which were predominantly mono- and di- nuclear are rounded, or spread, with extended cytoplasmic processes (fibroblast-like), identical to those described from bone marrow for rabbit [Fuller and Chambers, 1987], mouse [Domon and Wakita, 1991b; Domon and Wakita, 1991a] and human osteoclasts [Sarma and Flanagan, 1996] generated *in vitro*. In contrast to the many multinucleated cells obtained when cells are grown on plastic, very few large multinucleated cells appear on bone. Due to the unevenness of the bone, only some VR+ cells are in focus (Fig 1A). From 30 to 60% of the bone surface is covered with TRAP+ cells.

Characteristic resorption pits are present on bovine bone slices, in culture, by day 22, 24 and 31 days, by toluidine blue staining (Fig. 2A, C and D, respectively) and, confirmed by scanning electron microscopy (SEM) (Fig. 2B). Resorption pits are limited to a few areas per bone slice, and do not correspond to the larger number of VR+, TRAP+ cells on the bone. These findings suggest that our culture conditions on bone are conducive to a limited formation of functional osteoclasts, and, a more abundant preosteoclastic cell formation.

Because VR is one marker for preosteoclasts/osteoclasts, cells were examined on cytocentrifuge preparations for the acquisition of other osteoclast-related features - TRAP, NSE and

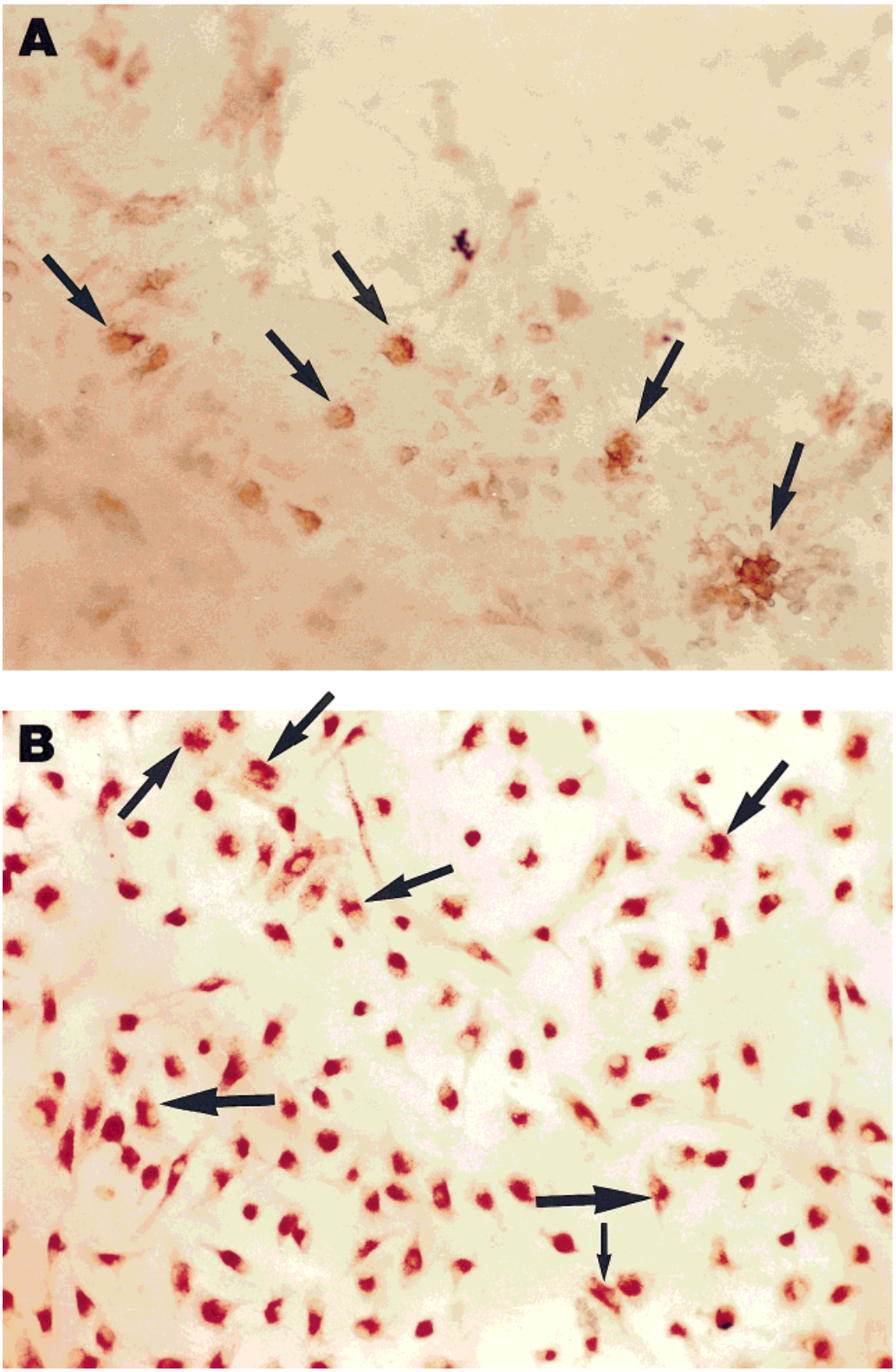

Fig. 1. Identification of VR and TRAP expressing cells on cultured bovine cortical bone slices. Mononuclear cells from leukapheresis product were grown for 11 days in αMEM+10% FBS on bone slices. Note the presence of VR **(A)**; and TRAP expressing cells **(B)** of mono- and low nuclearity (arrows point to some examples). For VR immunocytochemistry, absence of staining was demonstrated using isotype control antibodies (not shown). Photographs were obtained using a NIKON MICROPHOT-FXA microscope. 1.5 cm represents 50 μm.

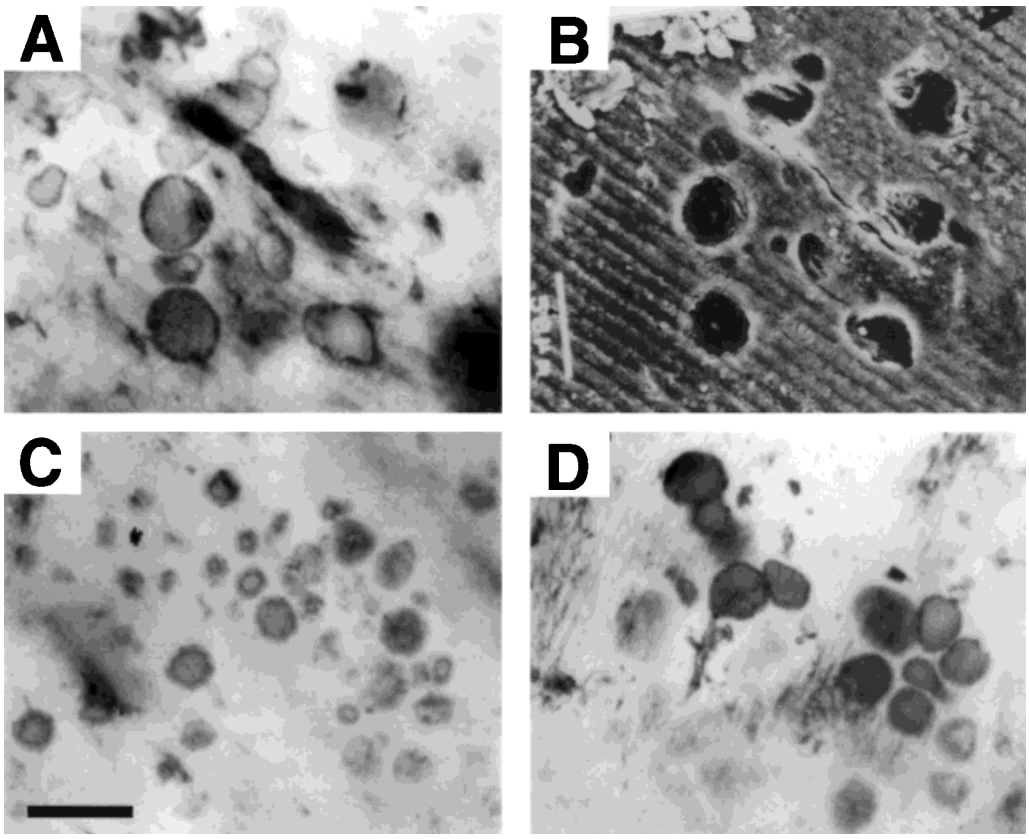

Fig. 2. Human osteoclasts from PBMC cultures form resorption pits on bone slices. Mononuclear cells, from heparinized peripheral blood, were grown on bone slices in αMEM+10% FBS. Osteoclast mediated pit formation is demonstrated on days 22 **(A)**, 24 **(C)**and 31 **(D)** by toluidine blue staining of bones, after removal of cells. Photographs were obtained using a NIKON MICROPHOT-FXA microscope. **B:** Scanning electron micrograph of pits from (A) demonstrates interruption of the saw marks made on the bone. Scale bar represents 50 μm.

CathK, beginning at culture initiation until about 2 weeks of culture. On day one, PBMC include monocytes and lymphocytes (Fig 3A) which are uniformly negative for TRAP (Fig 3B). The predominance of a monocyte derived population is confirmed by the presence of α-naphthyl-acetate-esterase (Fig 3C), that is NaF sensitive (NaF-S-NSE).

Three days later the cytoplasm of many monocytes is vacuolated, as massive cell death is occurring. A few tiny colonies of cells, remain on well washed plates, as well as large cell aggregates in the medium. Analysis of the adherent and non-adherent cells in culture, on cyocentrifuge preparations, reveals cells undergoing mitosis, as well as a small population of TRAP-/NSE- blasts (Fig 3D). The cytoplasm of a subset of cells is TRAP+ (Fig. 3E). Cells with the same cytomorphology as TRAP+ cells have NSE (Fig. 3F) activity, that is resistant to NaF inhibition (NaF-R-NSE). The variable staining of morphologically similar cells in these cultures for TRAP and NaF-R-NSE probably represents cells at different stages of maturity. TRAP is known to be an inducible enzyme, whose expression is associated with cell growth/activation and differentiation [reviewed in Drexler and Gignac, 1994]. TRAP and NaF-R-NSE activity increase with longer time in culture (compare Fig. 3B, E and H; and Fig. 3C, F and I). After day 3, cells with identical morphology stain for NaF-R-NSE and NSE, demonstrating the absence of typical monocytes.

By day 15, the adherent cells on plastic, are a homogeneous population of mono- and multinucleated cells (Fig. 3G), most of which stain intensely for TRAP (Fig. 3H), and NaF-R-NSE (Fig. 3I) (observed within cytoplasmic granules). Variable cytoplasmic staining suggests cells of differing maturity. It is not possible to distinguish between old cells, whose expression of NSE has declined and/or lost resistance to NaF, and, immature cells that are still forming. Non adherent lymphocytes are also present,

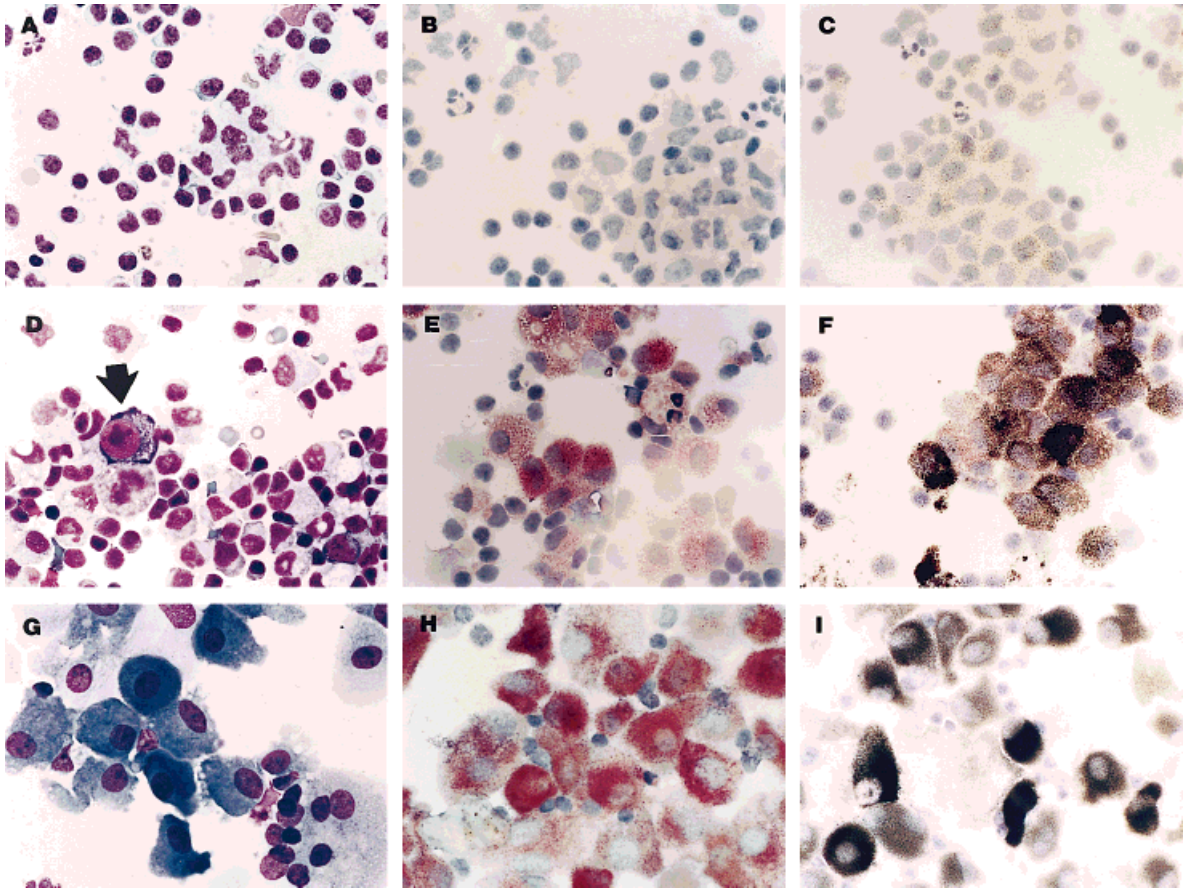

Fig. 3. The progression and development of cells with osteoclast markers, isolated from human blood leukapheresis product *in vitro*. Mononuclear cells, isolated from leukapheresis product by Ficoll/Hypaque separation, were seeded onto 24 well plates at 3×10^6 cells/ml at 1.5×10^6 cells/cm^2, and fed with αMEM+10% FBS every 2–3 days. At days 4 and 15, cells were mechanically removed from the plates, by gentle scraping, using a plastic pipette. Cells from days 1 (day of plating), 4 and 15, on cytocentrifuge preparations, were fixed with 2% paraformaldehyde, and stained with Wrights'/Giemsa for cell morphology **(A, D, G)**, TRAP **(B, E, H)**, and NaF-R-NSE **(C, F, I)**. Arrow in D points to a blast. The NSE of the monocytic population at day 1 is largely sensitive to NaF (C), but resistant cells, at days 4 and 15 stain dark brown to black (F and I). Photographs were obtained using a NIKON MICROPHOT-FXA microscope. 1.6 cm represents 50 µm.

demonstrated by CD3 and CD19 immunostaining (not shown).

In these cultures, multinuclear cells with well defined round borders, appear to arise by nuclear division without cytokinesis (Fig. 4A and B). Figure 4A is one example of several, showing a di-nuclear cell, with incompletely separated daughter nuclei, possibly at the end of nuclear division. Endomitosis is further illustrated in a larger multinucleated cell (Fig. 4B). These cells are indistinguishable morphologically from TRAP+, NaF-R-NSE, and VR+ cells on parallel cytocentrifuge preparations.

Figure 5 shows the time course of VR expression detected immunocytochemically, over a two week period. Cells on days 3 (not shown) and 6 (Fig. 5A) do not express detectable VRs. However, by day 8 (Fig. 5B), many mononucleated cells express VRs. By days 10, 13 (Fig. 5C and D), and 15 (Fig. 5E), mononuclear cells, dinuclear cells and multinucleated cells express VR. A control panel (Fig. 5F) shows no evidence of staining with an isotype control antibody on cells cultured for 15 days.

CathK is a cysteine protease expressed selectively and abundantly in osteoclasts [Drake et al., 1996]. Using affinity-purified anti-cathK antibodies (see Materials and Methods), Western blot analysis demonstrates a doublet at the expected Mr of about 30 kDa (Fig. 6). Proteins were isolated from cells cultured for different lengths of time - 20 days (Fig 6A, lane 2), and 7, 14, 21 and 24 days (Fig 6B). The upper band of the doublet seems to increase with time, when

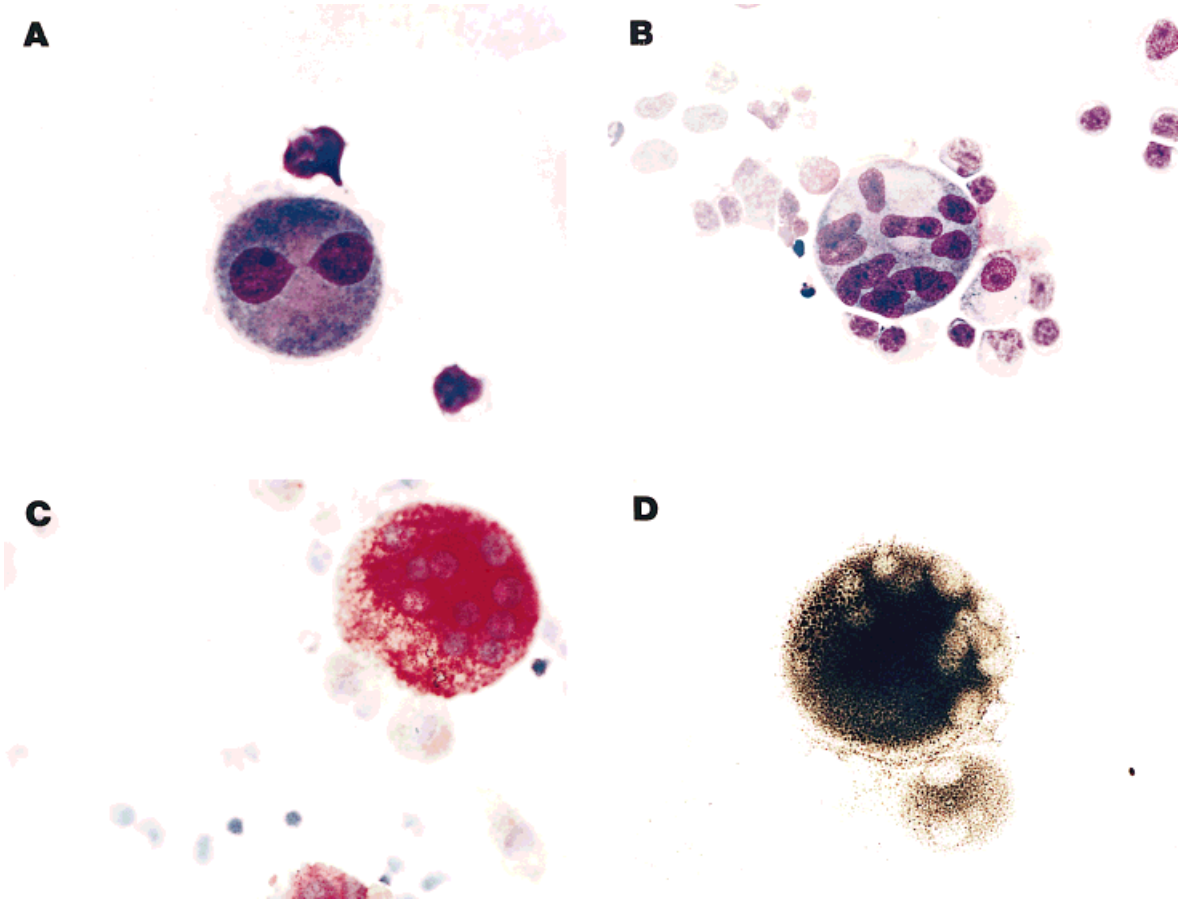

Fig. 4. Multinucleated cells found in culture have well-defined borders and stain intensely for Wright's/Giemsa, TRAP, and NaF-R-NSE. Wright's/Giemsa staining of a dinuclear **(A)**; and a multinuclear **(B)** cell. TRAP expression in a multinucleated cell with a well-defined border **(C)**. NaF-R-NSE is expressed abundantly in a multinucleated cell **(D)**. Endomitosis is suggested by the morphology of the nuclei in A and B as one possible mechanism for the generation of multinuclearity in preosteoclasts/osteoclasts. Photographs were obtained using a NIKON MICROPHOT-FXA microscope. 1.25 cm represents 25 µm.

compared to the lower band. The significance of this is unknown. Specificity of detection is ascertained by competition with peptide immunogen (Fig. 6A, lane 3), and, absence of reaction with proteins isolated at the time of plating (Fig. 6A, lane 1). A second experiment yielded similar results. Thus, this protein, like TRAP (Figure 3) and the VR (Figure 5), is induced within one week of culture.

One other marker, believed necessary for identifying cells of the osteoclast lineage, in culture (CR), is the membrane bound, calcitonin receptor [Suda et al., 1991]. Day 12 cells in these cultures exhibit specific ^{125} I -calcitonin binding and CR mRNA. Specific ^{125}I calcitonin binding is seen mainly over mononuclear, but also some multinucleated cells (Fig. 7A, B), with no cellular binding visible in the presence of excess unlabelled calcitonin (Fig. 7C, D). Furthermore, *in situ* hybridization using antisense mouse CR riboprobe demonstrates the presence of CR mRNA in these human cells (Fig. 7E F), and, osteoclast containing mouse cocultures [Lacey et al., 1995] (Fig. 7I and J). Cells in human (Fig. 7G, H) and mouse (Fig. 7K, L) control cultures show no evidence of hybridization, when sense CR probes are used.

Ribonuclease protection assays demonstrate cellular mRNA levels of osteoclast expressed genes, β3 subunit of VR, cathK, TRAP, c-src (an intracellular signal transduction molecule) [Soriano et al., 1991], and PU.1 (a transcription factor) [Tondravi et al., 1997], at days 1, 5, 9, 12, 15 and 18 of culture (Fig. 8). β3 and CathK mRNA levels are low at day 5, rising thereafter, consistent with the results of immunocytochemistry and Western blot analysis, respectively. The high level of β3 in control day 1 sample, is

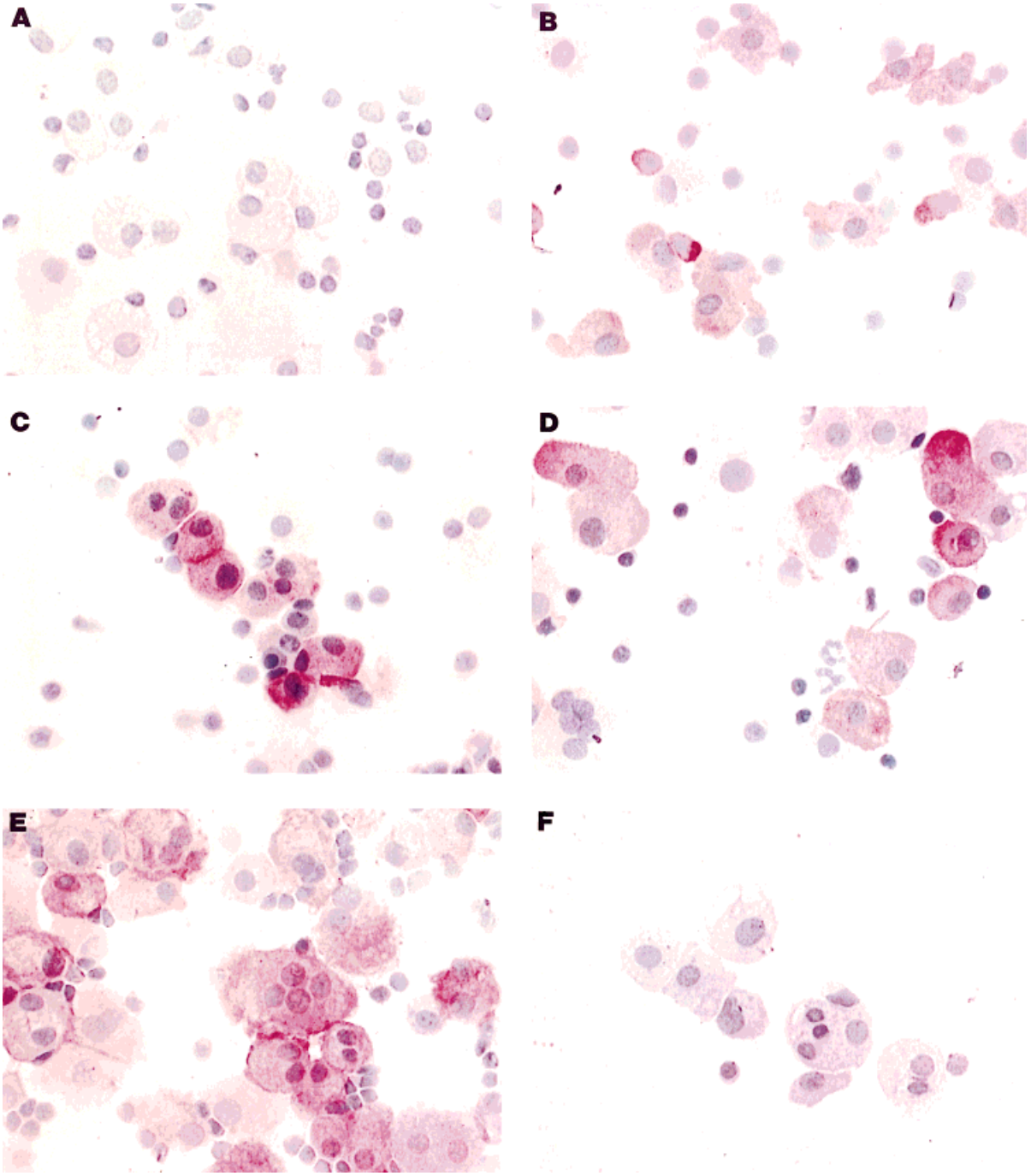

Fig. 5. Expression of VR by immunolocalization with 23c6 antibody, in cells forming in PBMC cultures. Mononuclear cells from leukapheresis product were grown for 3 weeks in αMEM+10% FBS. Cytocentrifuge preparations were prepared from day 6 **(A)**, 8 **(B)**, 10 **(C)**, 13 **(D)** and 15 **(E, F)** cultures, and fixed with acetone. Cells were then incubated with primary 23c6 antibody to the VR ($\alpha_v \beta_3$ integrin) or isotype control IgG1 antibody (F) at final concentrations of 10 mg/ml, and the antibody detected with AEC chromogen alkaline phosphatase substrate (LSAB 2 kit, Dako, Carpenteria, CA). Cells incubated with IgG1 isotype control were negative for 23c6 antibody membrane staining. 1.4 cm represents 50μm. Photographs were obtained using a NIKON MICROPHOT-FXA microscope.

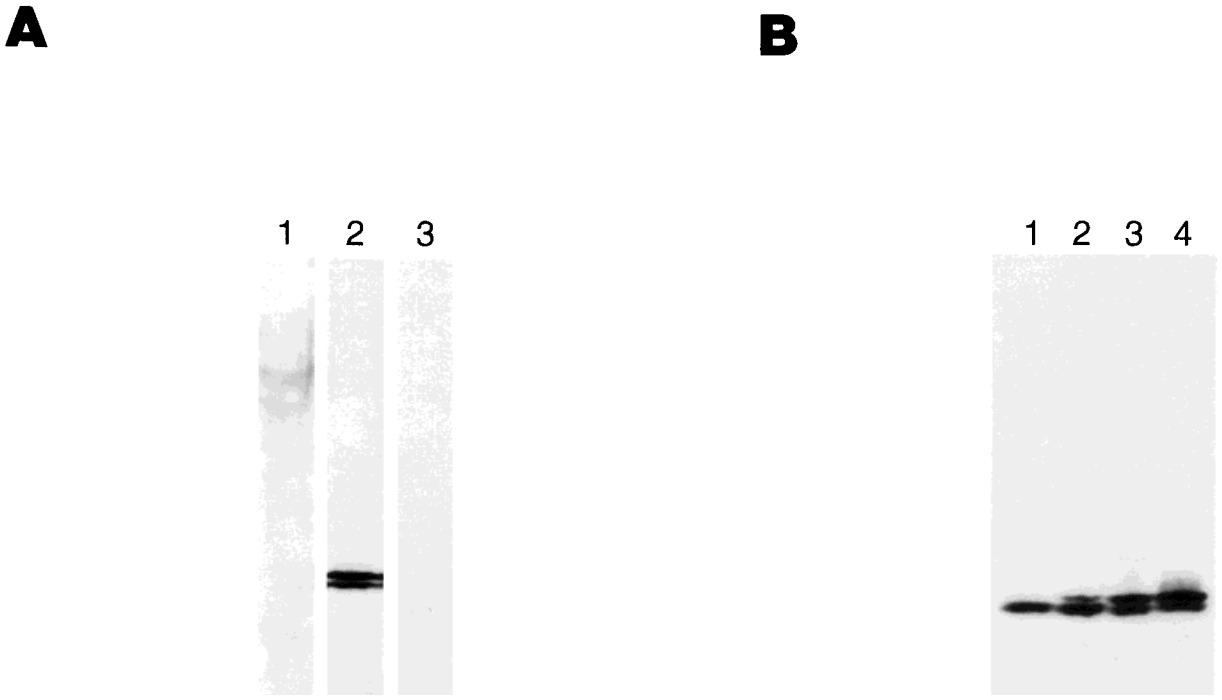

Fig. 6. CathK expression in human PBMC cultures. **A.** Proteins were isolated from cultures on day 1 **(lane 1)** or day 20 **(lanes 2 and 3)**. The Western blot was then probed with an affinity purified anti-peptide antibody to CathK (see Materials and Methods) in the absence (lane 1 and 2) or presence of competing peptide (lane 3). A doublet of specifically immunoreactive proteins is detected at a Mr of about 30 kd. **B.** Western blot showing the expression of CathK at different times in culture. The proteins at 30 kd in **lanes 1, 2, 3 and 4** represent antibody binding to proteins isolated on days 7, 14, 21 and 24.

consistent with the VR+ cells found in buffy coat preparations [Athanasou et al., 1990] that are lost during the first 5 days of culture. TRAP mRNA is absent at the time of plating but appears by day 5, consistent with the histochemical staining (see above). Both c-src [Soriano et al., 1991] and PU.1 [Tondravi et al., 1997] deficient animals develop osteopetrosis, due to a defect in osteoclast function, or formation, respectively. In the former case, osteoclasts form, but are incapable of resorbing bone, while PU.1 knockout animals lack osteoclasts and macrophages. PU.1 and c-src mRNA transcripts are present in cells at the time of plating, and subsequently, in adherent cells developing with preosteoclast/osteoclast phenotype. PU.1 and c-src mRNA levels did not change significantly with time in culture. These results were confirmed in a second experiment. Transcripts for OPGL, a recently described osteoclast differentiation factor [Lacey et al., 1998; Yasuda et al., 1998] were also present, most abundant at days 5 and 9, and declining thereafter.

DISCUSSION

In this study, we identify time-dependent changes in cell morphology and expression of osteoclast markers that, we propose, illustrate the progressive differentiation of early precursors into preosteoclasts and osteoclasts.

It has been reported that active human osteoclasts *in vivo* may have a low nuclei content [Kaye, 1984] similar to the cells we have described. Furthermore, the morphological, histochemical and/or immunostaining characteristics, that we have observed, are consistent with descriptions human osteoclasts *in vitro* cultured on bone from human [Sarma and Flanagan, 1996] and rabbit bone marrow [Fuller and Chambers, 1987]. Moreover, the human mononuclear osteoclast is identical to that of the mouse [Domon and Wakita, 1991a], when comparing transmission electron micrographs, (studies in progress), and, found to be positive for CR (by ^{125}I calcitonin binding, and *in situ* hybridization) (Fig. 7). These cells of low multinuclearity contrast with the larger multinucleated cells obtained in other PB [Fujikawa et al.,

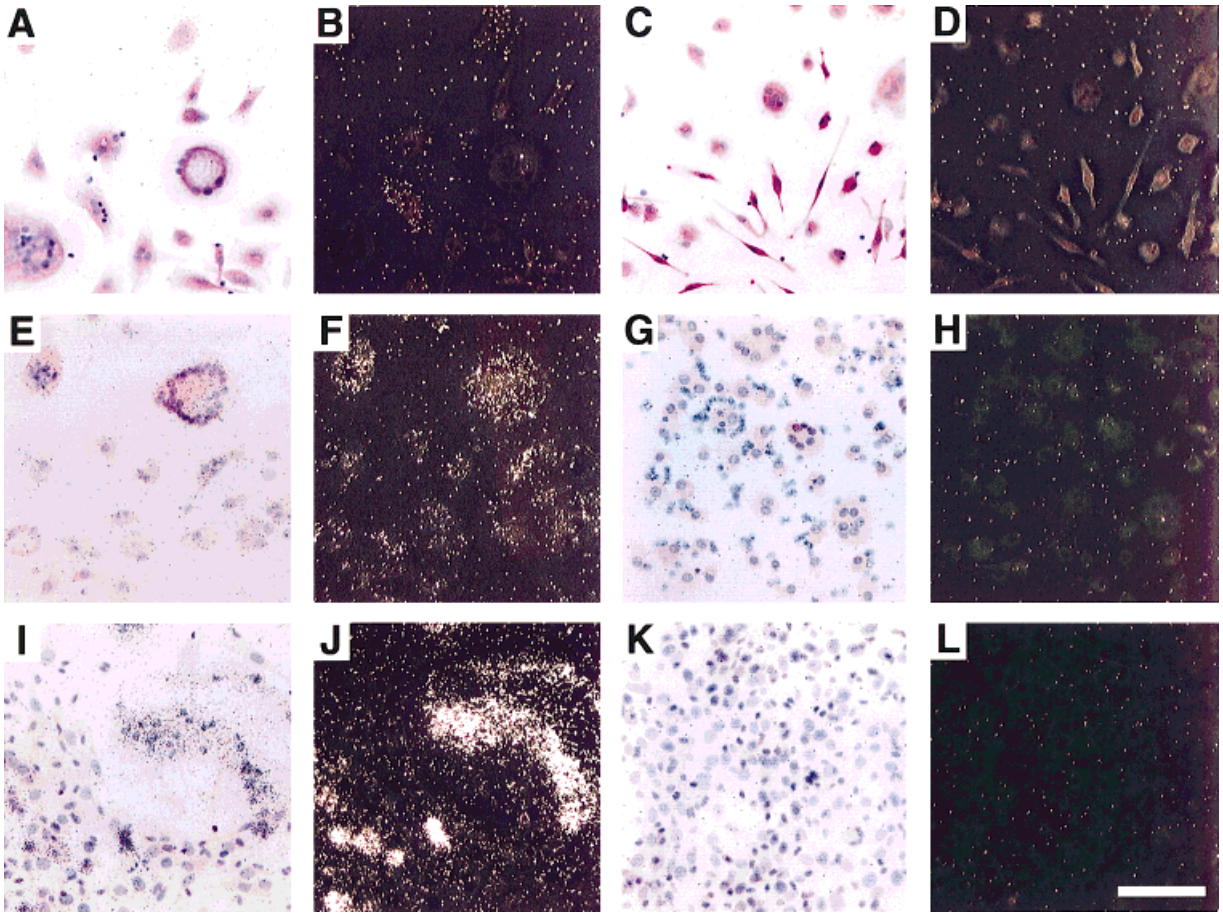

Fig. 7. Mononuclear and multinucleated cells formed in PBMC cultures demonstrate the presence of calcitonin receptors (CRs) and CR mRNA. PBMC, cultured for 12 days, demonstrate human ^{125}I calcitonin binding to mononuclear and some multinucleated cells (**A:** bright field; **B:** dark field). Cells in control cultures (incubated in the presence of excess cold calcitonin) show absence of radioligand binding (**C:** bright field; **D:** dark field). *In situ* hybridization to CR mRNA is shown with antisense (**E:** bright field; **F:** dark field), and sense human probes (**G:** bright field; **H:** dark field). *In situ* hybridization to CR mRNA with antisense (**I:** bright field; **J:** dark field), and sense mouse probes (**K:** bright field; **L:** dark field), in osteoclasts generated in 7 day cocultures of mouse marrow and ST2 cells. Scale bar represents 100 µm.

1996; Purton et al., 1996; Gregoretti et al., 1995], bone marrow [Roodman, 1995; Kassem et al., 1991] osteoclastoma [Grano et al., 1994], or cord blood [Roux et al., 1996; Orcel et al., 1990] systems, and also with many of the cells grown on plastic in this study.

It has been shown that osteoclast formation and activation are separable events, in rodents [Owens et al., 1996], and that stromal cells are not essential for the development of cells with osteoclast phenotype from one cord blood system [Roux et al., 1996] or PB [Matayoshi et al., 1996], although one other report for PB [Fujikawa et al., 1996] found them essential. It is not surprising, therefore, that resorption is limited, in the absence of stromal cells. The low level of resorption could reflect the few mature cells of osteoclast phenotype and low multinuclearity that develop on bone in the absence of stromal cells. The rest of the cells with osteoclast features may be preosteoclasts that have not yet completed differentiation due to insufficient stimuli; some of which can be provided by stromal cells. Alternatively, resorption could be mediated by the few larger multinucleated cells that form into osteoclasts.

In contrast to other PB osteoclast systems, our initial plating density is up to 100 fold higher [Matayoshi et al., 1996; Fujikawa et al., 1996], and this could account for the abundance of cells with an osteoclast phenotype. The threshhold for some necessary differentiation factor(s), for formation (such as CSF-1 and OPGL), may be met when cells are plated at

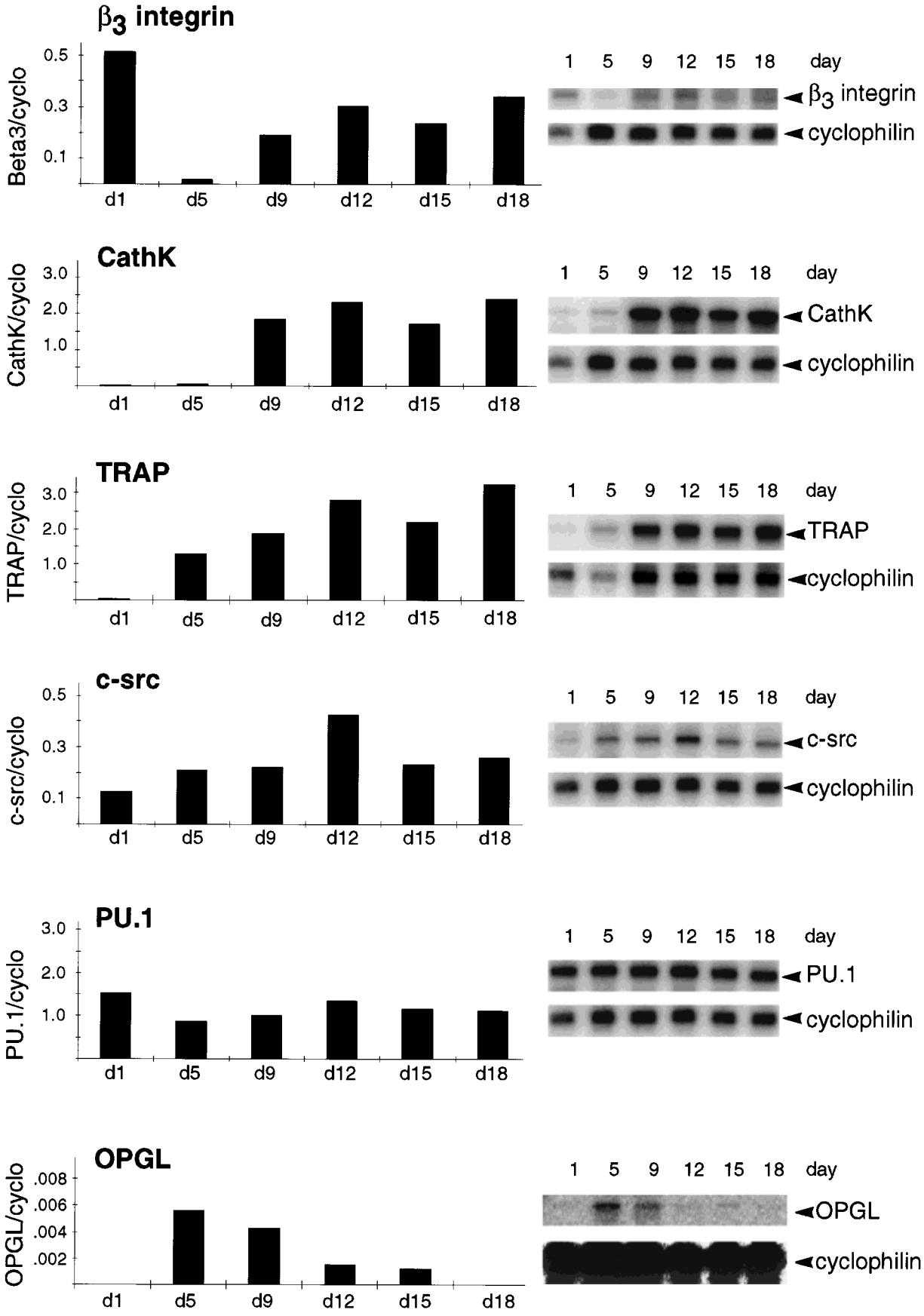

Fig. 8. Transcripts for osteoclast expressed genes accumulate in cells with preosteoclast/osteoclast phenotype, that arise from PBMC cultures. RNase protection assays were performed on total cellular RNA, isolated from PBMC cultures on days 1 (time of plating), 5, 9, 12, 15 and 18, with probes for osteoclast expressed genes, β3, CathK, TRAP, c-src, PU.1, and OPGL. Transcript levels were normalized to cyclophilin mRNA levels, assayed in each reaction as a control.

high density as in this study, since monocytes, macrophages and T-lymphocytes produce CSF-1, and T-lymphocytes, as well as adherent cells in this study, produce OPGL. Although less likely, it is also possible that factor-expressing endothelial cells are transferred with donor blood during collection.

The absence of TRAP in day 0 PBMC, and its appearance by day 4 in FBS containing medium in culture is consistent with the inducible nature of this enzyme during cell growth, activation and/or differentiation [Drexler and Gignac, 1994]. The induction of NaF-R-NSE expression, VR and cathK mRNA and protein, also occurs gradually and in parallel with the formation of TRAP+ cells in culture. This is consistent with reports that osteoclast precursor cells are masked in the monocyte fraction [Fujikawa et al., 1996]. The cells in our cultures do not exhibit NSE inhibition with the concentration of NaF that inhibits the monocyte enzyme, suggesting, that they are derived from specialized monocytes.

CR is expressed on the surface of a subset of human VR+ osteoclasts [Sarma and Flanagan, 1996], and, is present on mature mouse osteoclast precursors [Suda et al., 1992]. This is consistent with the presence of CR on, and CR mRNA in, cells with preosteoclast/osteoclast phenotype in this study (Fig. 7).

VR+ cells are present in low numbers in buffy coat preparations [Athanasou et al., 1990], consistent with β3 mRNA expression in day 1 samples. PU.1 is a myeloid and B-cell-specific transcription factor. PU.1 mRNA levels are elevated at the time of plating, likely due to the presence of many B lymphocytes; however, there are very few B cells associated with the adherent preosteoclast/osteoclast-like cells by day 5, and, from this time on, PU.1 mRNA levels reflect its presence in developing osteoclast lineage cells. Likewise, c-src mRNA levels, at the time of plating, likely reflect its presence in contaminating platelets and/or other cells, but by day 5, the contribution of c-src mRNA is made by adherent preosteoclast/osteoclast-like cells.

The question of endomitosis occurring in cells of the osteoclast lineage has been raised previously: In one study, freshly isolated rat osteoclasts were found to contain tetraploid nuclei [Zheng et al., 1991]. In another study, endomitosis was suggested by ^{3}H thymidine labelling experiments [Grano et al., 1994], using human osteoclastoma cells in culture. The morphology of the nuclei in Figure 4A and B strongly suggests that endomitosis may be one mechanism by which dinuclear and multinuclear cells arise in these cultures.

In conclusion, we have identified several markers that together demonstrate that human osteoclasts and their precursors can differentiate in cultures of PBMC within two weeks. Our conclusions are derived from thorough analyses of cell morphology, histochemistry, and immunocytochemistry performed every 2–3 days, from at least 4 independent experiments. We believe this *in vitro* system will facilitate the identification and characterization of factors promoting the differentiation and activation of human osteoclasts. This, in turn, will increase diagnostic and therapeutic options for osteolytic disease.

ACKNOWLEDGEMENTS

We thank Drs. Colin Dunstan and Sandy Marks Jr. for critically reviewing the manuscript, Tom Zamborelli and Marynette Rihanek for help in preparing the Cathepsin K antibody.

REFERENCES

Athanasou NA, Quinn J, Horton MA, McGee JO'D. 1990. New sites of cellular vitronectin receptor immunoreactivity detected with osteoclast-reacting monoclonal antibodies 13C2 and 23C6. Bone Miner. 8:7–22.

Baron R, Neff L, Van PT, Nefussi J-R, Vignery A. 1986. Kinetic and cytochemcial identifiction of osteoclast precursors and their differentiation into multinucleated osteoclasts. Am J Path 122:368–378.

Bossard MJ, Tomaszek TA, Thompson SK, Amegadzie BY, Hanning CR, Jones C, Kurdyla JT, Mcnulty DE, Drake FH, Gowen M, Levy MA. 1996. Proteolytic activity of human osteoclast cathepsin K. J Biol Chem 271:12517–12524.

Burgess TL, Ross SL, Qian Y-x, Brankow D, Hu S. 1995. Biosynthetic processing of *neu* differentiation factor. J Biol Chem 270:19188–19196.

Coccia PF, Krivit W, Cervenka J, Lawson C, Kersey JH, Kim TH, Nesbit ME, Ramsay NKC, Warketin PI, Teitelbaum SL, Kahn AJ, Brown DM. 1980. Successful bone marrow transplantation for infantile malignant osteopetrosis. New Engl J Med 302:701–708.

Connor JR, Dodds RA, James IE, Gowen M. 1995. Human osteoclast and giant cell differentiation: The apparent switch from nonspecific esterase to tartrate resistant acid phosphatase activity coincides with the in situ expression of osteopontin mRNA. J Histochem Cytochem 43: 1193–1201.

Domon T, Wakita M. 1991a. Electron microscopic and histochemical studies of the mononuclear osteoclast of the mouse. Am J Anat 192:35–44.

Domon T, Wakita M. 1991b. The three-dimensional structure of the clear zone of a cultured osteoclast. J Electron Microsc 40:34–40.

Drake FH, Dodds RA, James IE, Connor JR, Debouck C, Richardson S, Lee-Rykaczewski E, Coleman L, Rieman D, Barthlow R, Hastings G, Gowen M. 1996. Cathepsin K, but not cathepsins B, L, or S, is abundantly expressed in human osteoclasts. J Biol Chem 271:12511–12516.

Drexler HG, Gignac SM. 1994. Characterization and expression of tartrate-resistant acid phosphatase (TRAP) in hematopoietic cells. Leukemia 8:359–368.

Fujikawa Y, Quinn JMW, Sabokbar A, McGee JO'D, Athanasou NA. 1996. The human osteoclast precursor circulates in the monocyte fraction. Endocrinology 137:4058–4060.

Fuller K, Chambers TJ. 1987. Generation of osteoclasts in cultures of rabbit bone marrow and spleen cells. J Cell Physiol 132:441–452.

Grano M, Colucci S, de Bellis M, Zigrino P, Argentino L, Zambonin G, Serra M, Scotlandi K, Teti A, Zambonin Zallone A. 1994. New model for bone resorption study in vitro: Human osteoclast-like cells from giant cell tumors of bone. J Bone Miner Res 9:1013–1020.

Gregoretti MG, Bergui L, Aragno M, Cremona O, Marchisio PC, Caligaris-Capio F. 1995. Osteoclast precursors circulate in the peripheral blood of patients with aggressive multiple myeloma. Leukemia 9:1392–1397.

Hagenaars CE, van der Kraan AAM, Kawilarang-de Haas EWM, Visser JWM, Nijweide PJ. 1989. Osteoclast formation from cloned pluripotent hemopoietic stem cells. Bone Miner 6:179–189.

Hattersley G, Chambers TJ. 1989. Generation of osteoclasts from hemopoietic cells and a multipotential cell line in vitro. J Cell Physiol 140:478–482.

James IE, Dodds RA, Lee-Rykaczewski E, Eichman CF, Connor JR, Hart TK, Maleeff BE, Lackman RD, Gowen M. 1996. Purification and characterization of fully functional human osteoclast precursors. J Bone Miner Res 11:1608–1618.

Kahn AJ, Simmons DJ. 1975. Investigation of the cell lineage in bone using a chimera of chick and quail embryonic tissue. Nature 258:325–327.

Kassem M, Mosekilde L, Rungby J, Melsen F, Eriksen EF. 1991. Formation of osteoclasts and osteoblast-like cells in long-term human bone marrow cultures. APMIS 99:262–268.

Kaye M. 1984. When is it an osteoclast? J Clin Pathol 37:398–400.

Lacey DL, Erdmann JM, Teitelbaum SL, Tan H-L, Ohara J, Shioi A. 1995. Interleukin 4, Interferon- and Prostaglandin E impact the osteoclastic cell-forming potential of murine bone marrow macrophages. Endocrinology 136: 2367–2376.

Lacey DL, Timms E, Tan H-L, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Caparelli C, Eli A, Qian Y-x, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ. 1998. Osteoprotegerin (OPG) ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93:165–176.

Marks SC, Popoff SN. 1988. Bone cell biology: The regulation of development, structure and function in the skeleton. Am J Anat 183:1–44.

Matayoshi A, Brown C, DiPersio JF, Haug J, Abu-Amer Y, Liapis H, Kuestner R, Pacifici R. 1996. Human blood-mobilized hematopoietic precursors differentiate into osteoclasts in the absence of stromal cells. Proc Natl Acad Sci USA 93:10785–10790.

Orcel P, Bielakoff J, de Vernejoul M-C. 1990. Formation of multinucleated cells with osteoclast precursor features in human cord monocytes cultures. Anat Rec 226:1–9.

Owens JM, Gallagher AC, Chambers TJ. 1996. Bone cells required for osteoclastic resorption but not for osteoclastic differentiation. Biochem Biophys Res Commun 222: 225–229.

Purton LE, Lee MY, Torok-Storb B. 1996. Normal human peripheral blood mononuclear cells mobilized with granulocyte colony-stimulating factor have increased osteoclastogenic potential compared to nonmobilized blood. Blood 87:1802–1808.

Roodman GD. 1995. Application of bone marrow cultures to the study of osteoclast formation and osteoclast precursors in man. Calcif Tissue Int 56(Suppl 1):S22-S23.

Roux C, Ravaud P, Cohen-Solal M, de-Vernejoul MC, Guillemant S, Cherruau B, Delmas P, Dougados M, Amor B. 1994. Biologic, histologic and densitometric effects of oral risedronate on bone in patients with multiple myeloma. Bone 15:41–49.

Roux S, Quinn J, Pichaud F, Orcel P, Chastre E, Jullienne A, de Vernejoul M-C. 1996. Human cord blood monocytes undergo terminal osteoclast differentiation in vitro in the presence of culture medium conditioned by giant cell tumor of bone. J Cell Physiol 168:489–498.

Sarma U, Flanagan AM. 1996. Macrophage colony-stimulating factor induces substantial osteoclast generation and bone resorption in human bone marrow cultures. Blood 88:2531–2540.

Sheven BAA, Visser JWM, Nijwiede PJ. 1986. In vitro osteoclast generation from different marrow fractions including a highly enriched haemopoietic stem cell population. Nature 321:79–81.

Soriano P, Montgomery C, Geske R, Bradley A. 1991. Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. Cell 64:693–702.

Suda T, Takahashi N, Martin TJ. 1992. Modulation of osteoclast differentiation. Endocrine Reviews 13:66–80.

Sun T. 1985. Atlas of cytochemistry and immunochemistry of hematologic neoplasms. Chicago: American Society of Clinical Pathologists Press. pp. 34,111.

Tondravi MM, McKercher SR, Anderson K, Erdmann JM, Quiroz M, Maki R, Teitelbaum SL. 1997. Osteopetrosis in mice lacking haematopoietic transcription factor PU.1. Letters to Nature 386:81–84.

Walker DG. 1973. Osteopetrosis cured by temporary parabiosis. Science 180:875.

Walker DG. 1975. Control of bone resorption by hematopoietic tissue. The induction and reversal of congenital osteopetrosis in mice through the use of bone marrow mononuclear phagocytes. J Exp Med 142:651–663.

Wilcox JN. 1993. Fundamental principles of in situ hybridization. J Histochem 41:1721–1723.

Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T. 1998. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci USA 95:3597–3602.

Zheng MH, Papadimitriou JM, Nicholson GC. 1991. A quantitative cytochemical investigation of osteoclasts and multinucleate giant cells. Histochem J 23:180–188.