

THE HUMAN OSTEOCLAST PRECURSOR CIRCULATES IN THE MONOCYTE FRACTION

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ABSTRACT: The osteoclast is known to be formed by fusion of circulating mononuclear precursor cells of haematopoietic origin. The precise nature of these circulating cells and, in particular, their relation to monocytes is unknown. We have developed an *in vitro* system of human osteoclast formation whereby human monocytes [CD14, CD11a, CD11b and HLA-DR positive, and tartrate-resistant acid phosphatase (TRAP), calcitonin receptor (CTR), vitronectin receptor (VNR) negative] were isolated and cocultured for up to 21 days with UMR106 rat osteoblast-like cells or ST2 mouse preadipocytic bone marrow stromal cells in the presence of $1\alpha,25$ dihydroxyvitamin D₃ ($1,25(\text{OH})_2\text{D}_3$) and macrophage colony stimulating factor (M-CSF). Numerous TRAP, VNR and CTR positive multinucleated cells, capable of extensive lacunar bone resorption, formed in these cocultures; the absolute requirements for this to occur were contact with the above bone stromal cells, $1,25(\text{OH})_2\text{D}_3$, and M-CSF. These results show that the human mononuclear osteoclast precursor circulates in the monocyte fraction and exhibits a monocyte phenotype, acquiring osteoclast phenotypic features in the process of differentiation into mature functional osteoclasts.

Osteoclasts are multinucleated cells responsible for bone resorption. They are derived from the pluripotential hematopoietic stem cell (1) and share more committed hematopoietic progenitors with cells of the mononuclear phagocyte system (MPS) (2). However, the precise details of the osteoclast cell lineage are uncertain; in particular, the exact nature of the circulating mononuclear precursor cells, which fuse to form osteoclasts, and their relationship to monocytes has not been established. In addition, the cellular and hormonal requirements for human osteoclast differentiation are not known. In this study, we have addressed these issues by developing an *in vitro* system for the generation of large numbers of human osteoclasts from peripheral blood mononuclear cells.

MATERIALS AND METHODS

Preparation of cells and method of coculture:

Monocytes were isolated from the peripheral blood of 20 normal subjects. Blood was diluted 1:1 in Hanks Balanced Salt Solution (HBSS), layered over Ficoll Hypaque, and centrifuged (693g), then washed and resuspended in HBSS. 5×10^5 peripheral blood mononuclear cells (PBMCs) were then added to human cortical bone slices (10mm^2) or 6mm diameter glass coverslips, on half of which 4×10^4 bone stromal cells [osteoblast-like UMR106 or UMR106.01 cells derived from rat osteosarcoma cells (TJ Martin, Melbourne, Australia), or mouse preadipocytic bone marrow stroma-derived ST2 cells (RIKEN cell bank, Japan)] had previously been cultured for 24 hours in alpha minimal essential medium with 10% added foetal calf serum (MEM-FCS) (Gibco, UK). After 1 hour, cultures were rinsed in medium to remove non-adherent cells, then maintained in 1ml MEM-FCS for up to 21 days, both in the presence and absence of $1,25(\text{OH})_2\text{D}_3$ (10^{-7}M) (Solvay Duphar, NL), recombinant human M-CSF (25ng/ml) (R&D Systems, UK) and

dexamethasone 10^{-8}M (Sigma, UK). In all cultures, medium (with added factors) was entirely replaced every three days.

Determination of osteoclast characteristics:

Following incubation for 24 hours, 7, 14 and 21 days, cocultures on coverslips were fixed and stained histochemically for tartrate-resistant acid phosphatase (TRAP) (3); using an indirect immunoperoxidase technique they were also immunocytochemically stained for the highly osteoclast-associated vitronectin receptor (VNR) (4), and the macrophage-associated antigens CD11a, CD11b, CD14, and HLA-DR with the monoclonal antibodies: 23C6, MH24, TMG6-5, GRS1, and CR3/43 respectively (5). Cultures were also assessed after 24 hours and at day 14 for the presence of CT receptors by autoradiography using ¹²⁵I-labelled salmon calcitonin ligand binding as previously described (6). Bone resorption by cultured cells on cortical bone slices was assessed after 24 hours, 7 days, 14 days and 21 days by scanning electron microscopy (SEM) as previously described (7).

Evaluation of the requirements for osteoclast differentiation:

To estimate the proportion of unfractionated peripheral blood mononuclear cells (PBMCs) differentiating into lacunar bone-resorbing cells, a suspension of PBMCs (5×10^6 cells/ml) was sequentially diluted in MEM-FCS to produce a suspension containing 5×10^5 , 5×10^4 , 5×10^3 , 5×10^2 , 5×10^1 cells per ml. 100µl of each of these suspensions was then added to wells containing bone slices which had previously been seeded with UMR106 cells. After 21 days incubation, the bone slices were assessed by SEM for evidence of lacunar bone resorption.

To determine whether a specific stromal cell influence is required for osteoclast formation, monocytes were also cocultured for 21 days in the presence of $1,25(\text{OH})_2\text{D}_3$, dexamethasone and M-CSF with the following stromal cell

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lines: mouse fibroblast-like L929 cells, and human osteoblast-like MG63 and SaOS-2 cells. To determine whether UMR106 cells release a soluble factor that stimulates differentiation of monocytes into bone-resorbing cells, 5×10^5 monocytes were added to bone slices devoid of stromal cells and cultured in the presence of $1,25(\text{OH})_2\text{D}_3$, dexamethasone and M-CSF, together with UMR106-conditioned medium or UMR106 cells seeded onto a millipore filter (pore size $0.45 \mu\text{m}^2$) (Becton Dickinson, UK); the pores of the filters were large enough to allow transfer of soluble factors but not physical contact between the UMR106 cells and monocytes. These bone slices were cultured for 21 days before processing for SEM.

RESULTS: Isolated PBMCs from all samples were shown by histochemistry, immunohistochemistry and ^{125}I -labelled salmon calcitonin ligand binding respectively to be entirely negative for the following osteoclast markers: TRAP, VNR, and CTR. Immunohistochemical staining showed that these cells expressed the following mononuclear phagocyte antigens which are known not to be present on osteoclasts: CD11a, CD11b, CD14 and HLA-DR (5). In addition, these cells, when cultured for 24 hours in the presence or absence on bone slices of all the above stromal cells showed no evidence of lacunar bone resorption by SEM. These cells thus exhibited the phenotypic characteristics of monocytes and not osteoclasts.

Numerous TRAP, VNR and CTR positive multinucleated cells were present on coverslips upon which monocytes from all samples had been cocultured for 14 days with UMR106, UMR106.01 or ST2 cells in the presence of $1,25(\text{OH})_2\text{D}_3$ and M-CSF (Figure 1). CD11a, CD11b, CD14 and HLA-DR positive mononuclear and multinucleated cells were also present in these cocultures but a number of multinucleated cells were noted to be negative for these antigens. Monocytes from all blood samples, when cocultured under these conditions with UMR106.01, UMR106 or ST2 cells also showed functional evidence of osteoclast differentiation with formation of numerous resorption lacunae on all bone slices. This was first seen as single pits in 7 day cocultures and in 14 and 21 day cultures as extensive areas of lacunar resorption (more than 25 resorption pits on each bone slice) (Figure 2). Over half the bone slices examined contained more than 200 resorption pits and several bone slices showed resorption of up to 25% of the bone slice area. Serial dilution of the leucocyte suspension added to bone slices showed that resorption lacunae were formed when as few as 500 cells were added to each well (i.e. approximately 50 adherent monocytes per bone slice).

When either M-CSF or $1,25(\text{OH})_2\text{D}_3$ was omitted, TRAP, VNR and CTR positive multinucleated cells and bone resorption were not seen in monocyte/UMR106 or monocyte/ST2 cocultures. The omission of dexamethasone greatly reduced but did not abolish the above evidence of

osteoclast differentiation. Cocultures of monocytes with L929, MG63 or SaOS-2 cells showed no evidence of bone resorption. VNR positive multinucleated cells were only seen when monocytes were cocultured with UMR106, UMR106.01 or ST2 cells; occasional VNR positive mononuclear cells were found in cocultures of monocytes with all stromal cell types even in the absence of $1,25(\text{OH})_2\text{D}_3$ and M-CSF.

Lacunar resorption was not seen in 21 day cocultures where monocytes incubated alone on bone slices were separated from UMR106 cells by a membrane filter, i.e. were not in direct cell-cell contact. Addition of conditioned medium from UMR106 cells to human monocytes cultured alone on bone slices, (both in the presence and absence of $1,25(\text{OH})_2\text{D}_3$ and M-CSF) also did not result in lacunar resorption. When monocytes were cultured on coverslips and bone slices for 3 days prior to the addition of UMR106 cells, osteoclastic differentiation and extensive lacunar resorption was still evident.

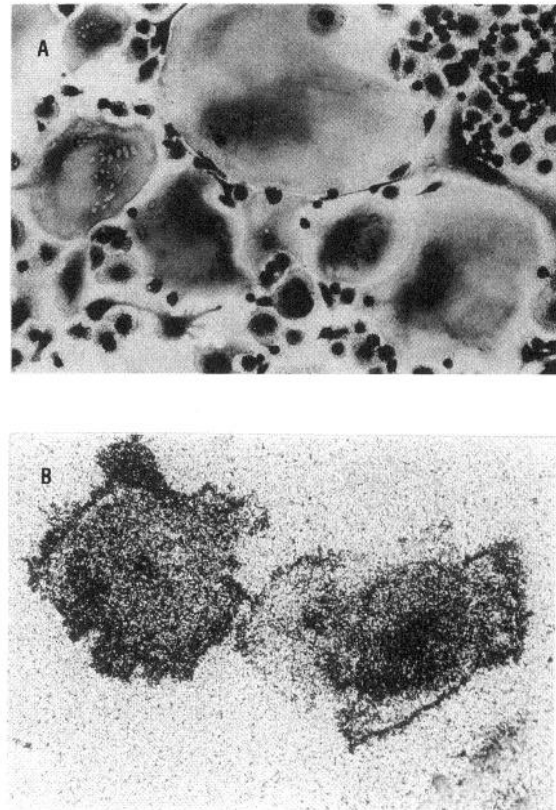


Figure 1. Monocyte/UMR106.01 coculture after 14 days incubation showing: (a). Multinucleated cells positive for TRAP (x 200). (b). the presence of CT receptors on multinucleated cells by autoradiography using ^{125}I labelled CT (x 400).

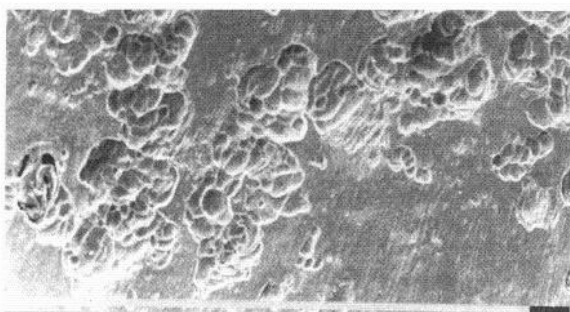


Figure 2. Extensive lacunar resorption of human cortical bone slice seen in monocyte/UMR106.01 cocultures after 21 days incubation (white bar = 1 mm).

DISCUSSION: Formation of multinucleated cells exhibiting the osteoclast phenotypic characteristics of TRAP, VNR, CTR and the ability to form resorption lacunae was only noted when cells of the monocyte fraction were cultured in contact with bone stromal cells of a specific type (i.e. UMR106 and ST2 cells) in the presence of $1,25(\text{OH})_2\text{D}_3$ and M-CSF. Although osteoclast formation from mouse monocytes occurs when these cells are cocultured with ST2 or UMR106 cells without addition of M-CSF (8,9), M-CSF is known to be essential for the proliferation and differentiation of mouse haemopoietic osteoclast precursors (10). Osteoclast formation from human monocytes may require human M-CSF to be added to this coculture system as rodent M-CSF (which would be secreted by ST2 and UMR106 cells) does not bind to the human M-CSF receptor (11).

The osteoclast has highly specific cytochemical, antigenic and ultrastructural phenotypic characteristics and is, uniquely, capable of lacunar bone resorption (12). On this basis it has been proposed that the osteoclast precursor cell, both in hematopoietic tissues and in the circulation, is distinct from that of cells of the monocyte/macrophage lineage. Our results show, however, that mononuclear osteoclast precursors not only circulate in the monocyte fraction but also express the phenotypic characteristics of monocytes and not osteoclasts. Moreover, they show that the proportion of cells in the monocyte fraction capable of osteoclast differentiation is at least 2%. These features would argue against osteoclasts being formed from a distinct population of osteoclast precursors alone.

Cultured PBMCs of the monocyte fraction only acquired osteoclast phenotypic features after they had been cultured (in the presence of $1,25(\text{OH})_2\text{D}_3$ and M-CSF) in contact with bone-derived stromal cells. This suggests that osteoclastic differentiation of these circulating precursors occurs in bone itself, where all these factors would be present in the bone microenvironment. The *in vitro* system described in this study would appear to recapitulate this microenvironment,

and should provide a useful model for the investigation of human osteoclast generation in health and disease.

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