Human osteoclast formation and bone resorption by monocytes and synovial macrophages in rheumatoid arthritis

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Abstract

Objective—To determine whether synovial macrophages and monocytes isolated from patients with rheumatoid arthritis are capable of differentiating into osteoclastic bone resorbing cells; and the cellular and humoral conditions required for this to occur.

Methods—Macrophages isolated from the synovium and monocytes from the peripheral blood of rheumatoid arthritis patients were cultured on bone slices and coverslips, in the presence and absence of UMR 106 rat osteoblast-like cells, 1,25-dihydroxy vitamin D₃ (1,25(OH)₂D₃) and macrophage colony stimulating factor (M-CSF), and assessed for cytochemical and functional evidence of osteoclast differentiation.

Results—Isolated calcitonin receptor (CTR), tartrate resistant acid phosphatase (TRAP), and vitronectin receptor (VNR) negative, CD11b and CD14 positive monocytes and macrophages differentiated into CTR, TRAP, and VNR positive multinucleated cells capable of extensive lacunar bone resorption when co-cultured for 14 d with UMR 106 cells in the presence 1,25(OH)₂D₃ and M-CSF.

Conclusions—Mononuclear phagocytes (monocytes and macrophages) from rheumatoid arthritis patients are capable of differentiating into multinucleated cells showing all the cytochemical and functional criteria of mature osteoclasts. Synovial macrophage-osteoclast differentiation may represent an important cellular mechanism in the bone destruction associated with rheumatoid arthritis.

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Rheumatoid arthritis is a chronic inflammatory disease of unknown aetiology that results in progressive damage to joint tissues. There is extension of exuberant inflamed synovial tissue (pannus) over the articular surface, leading to its destruction.¹ Rheumatoid pannus also extends into juxta-articular bone, producing marginal erosions and cyst-like areas of bone destruction. In the inflamed synovium from which pannus develops, there is marked thickening of the synovial intima, which is composed largely of lining cells of macrophage phenotype²⁻⁵; there is also a heavy subintimal chronic inflammatory cells infiltrate which includes numerous lymphocytes, plasma cells,

and macrophages.^{3,6,7-9} Both intimal and subintimal synovial macrophages in rheumatoid arthritis are derived from the circulation, blood monocytes passing through the tall endothelium of postcapillary venules to enter the synovial tissues.¹⁰

Macrophages rather than T cells have been found to predominate at the articular margins where there is bone and cartilage destruction.^{11,12} A significant correlation has been reported between the degree of joint erosion and the number of synovial macrophages.¹³ At the site of marginal erosions, bone resorption, however, appears to be effected largely by recognisable osteoclasts.¹⁴ Various macrophage derived cytokines are known to enhance osteoclastic bone resorption indirectly (through osteoblast stimulation)¹⁵ and several of these cytokines also promote the release of collagenase, elastase, plasminogen activator, and prostaglandins by resident cells of bone and joint,^{16,17} factors which may play a role in bone destruction in rheumatoid arthritis.¹⁸

Several recent animal studies have shown that monocytes and macrophages (including those isolated from tumours and inflammatory lesions) are capable of differentiating into osteoclastic bone resorbing cells when co-cultured with bone derived stromal cells in the presence of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃)¹⁹⁻²²; the mononuclear and multinucleated cells formed in these co-cultures show all the phenotypic characteristics of osteoclasts, including expression of tartrate resistant acid phosphatase (TRAP), calcitonin receptors (CTR), and most importantly, the functional ability to produce resorption lacunae in bone. In the context of an inflammatory disease such as rheumatoid arthritis, where there is a heavy macrophage infiltrate in synovial tissues, osteoclast differentiation by monocytes and macrophages could represent an important mechanism for the pathogenesis of bone destruction. In this study we have sought to determine whether this cellular mechanism of osteoclast formation and pathological bone resorption operates in rheumatoid arthritis. Peripheral blood monocytes and synovial macrophages from rheumatoid arthritis patients were co-cultured with osteoblastic cells in the presence and absence of humoral factors which are known to be important in mouse osteoclast formation, in order to determine the cellular and humoral conditions for mononuclear phagocyte-osteoclast differentiation in rheumatoid arthritis.²³⁻²⁵

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Methods

Synovial tissue was obtained fresh from six patients with seropositive rheumatoid arthritis,²⁶ all of whom were undergoing knee arthroplasty operations. The synovial samples were obtained from five women and one man (age range 51 to 82 years).

Monocytes were isolated from the peripheral blood of eight patients with seropositive rheumatoid arthritis; seven women and one man were studied (age range 46 to 74 years).

ISOLATION AND CULTURE OF MONOCYTES AND SYNOVIAL MACROPHAGES

For isolation of monocytes, blood was diluted 1:1 in Hanks' balanced salt solution (HBSS) (Gibco, UK), layered over Ficoll Hypaque (Pharmacia), centrifuged, washed, and then resuspended in 5 ml of α minimal essential medium (MEM) (Gibco, UK) with 10% fetal calf serum (Gibco, UK) containing glutamine, benzylpenicillin, and streptomycin (MEM/FCS). The number of cells in the resultant suspension was counted in a haemocytometer. Peripheral blood mononuclear cells (5×10^5) were then added to 7 mm diameter wells of 96-well plates containing human cortical bone slices (5 mm²), prepared as previously described,²⁷ or 6 mm diameter glass coverslips, on half of which 4×10^4 osteoblast-like UMR 106 cells—a rat osteosarcoma cell line²⁸—had previously been cultured for 24 h in MEM/FCS.

Specimen radiography was carried out on all samples of rheumatoid arthritis synovium to determine whether any bone particles were present in the synovial membrane; blocks were also taken for routine histology. The remainder of the synovial specimen was then cut into small pieces (approximately 1 mm³) and washed thoroughly in HBSS. The synovial fragments were digested in HBSS containing 1 mg ml⁻¹ collagenase type I (Sigma, UK) and 0.25% trypsin for 90 min at 37°C. The suspension was filtered through a 70 μ m cell strainer (Falcon, UK) before being centrifuged at 680 *g* for 10 min. The cell pellet was resuspended in 5 ml MEM/FCS and the cell suspension (1×10^5 cells/well) was then added

to bone slices and to glass coverslips, half of which had been preseeded with UMR 106 cells as described above.

The monocyte-UMR 106 and macrophage-UMR 106 co-cultures were maintained in 24-well plates, in 1 ml MEM/FCS for up to 14 days, both in the presence and absence of 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ (Solvay Duphar, The Netherlands), 25 ng ml⁻¹ human macrophage colony stimulating factor (M-CSF) (R & D Systems, UK), and 10^{-8} M dexamethasone. In all cultures, the medium (with added factors) was entirely replaced every 3 days.

As controls, cultures of monocytes and synovial macrophages were also set up with and without UMR 106 cells, and in the presence or absence of $1,25(\text{OH})_2\text{D}_3$, M-CSF, and dexamethasone.

CYTOCHEMICAL AND IMMUNOCYTOCHEMICAL CHARACTERISATION OF ISOLATED AND CULTURED CELLS

Isolated monocytes and macrophages which had been cultured on coverslips for 24 hours and 7 days were fixed in citrate acetone and stained for tartrate resistant acid phosphatase (TRAP), an osteoclast associated enzyme,²⁹ using a kit from Sigma. For immunocytochemistry, cell cultures on coverslips were fixed in cold acetone and stained by an indirect immunoperoxidase technique³⁰ for the detection of CD51, the vitronectin receptor (VNR), a highly osteoclast associated antigen, using monoclonal antibody 23C6 (a kind gift of Professor M Horton, London).³¹ Cultures on coverslips were also stained immunohistochemically for the presence of CD11b and CD14, monocyte/macrophage antigens known not to be expressed by human osteoclasts,³¹ with the monoclonal antibodies TMG6-5 and MEM-18 respectively. These monoclonal antibodies were derived from the 5th International Workshop on Human Leucocyte Differentiation Antigens.³³

DETECTION OF CALCITONIN RECEPTORS ON MONOCYTE CULTURES

Peripheral blood mononuclear cells isolated and cultured on glass coverslips in the presence and absence of UMR 106 cells, $1,25(\text{OH})_2\text{D}_3$, and M-CSF were assessed after incubation for 24 hours and 14 days for the presence of calcitonin receptors (CTR) by autoradiography using ¹²⁵I-labelled salmon calcitonin ligand binding, as previously described.³⁴ Briefly, peripheral blood mononuclear cells and UMR 106 cells were co-cultured on glass coverslips for one hour at 22°C in MEM/FCS containing 0.15% bovine serum albumin (BSA) with 0.25 μ Ci ¹²⁵I-calcitonin (Amersham International, Aylesbury, UK). After allowing labelled calcitonin to bind for two hours, the cells were washed with cold MEM, fixed for 10 minutes in 2% glutaraldehyde-10% formalin solution, and air dried. Non-specific binding was assessed in the presence of an excess amount of unlabelled calcitonin (300 nM). The coverslips were then dipped in K-5 photographic emulsion and processed for autoradiography. Negative controls consisted of UMR 106 cells alone; positive controls consisted of adherent cultured rabbit osteoclasts.

Figure 1 Rheumatoid synovial macrophage/UMR 106 co-culture after 14 d incubation, showing multinucleated cells positive for TRAP ($\times 200$).

Figure 2 Lacunar resorption of human cortical bone slices seen in rheumatoid monocyte/UMR 106 co-cultures after 14 d incubation (black bar = 100 μ m).

FUNCTIONAL ASSESSMENT OF THE ABILITY OF CULTURED CELLS TO CARRY OUT LACUNAR BONE RESORPTION

Rheumatoid arthritis monocytes and macrophages incubated on bone slices in the presence and absence of UMR 106 cells and the humoral factors described above were cultured for periods of 24 hours, 7 days, and 14 days. To determine whether lacunar resorption of bone slices had occurred, confluent cell cultures were removed from the surface of the bone in the following manner: each bone slice was rinsed in phosphate buffered saline containing 0.2% EDTA, then placed in 0.25% trypsin solution for 10 min, after which they were rinsed vigorously in distilled water and immersed in 0.25 M ammonium hydroxide overnight; they were then washed in distilled water, dehydrated in graded alcohols, and air dried. The bone slices were mounted onto aluminium stubs using double sided Sellotape, sputtered with gold, and examined in a Phillips SEM 505 scanning electron microscope. The presence or absence of resorption pits on the

bone surface was noted,³⁵ and the extent of resorption determined semiquantitatively.

Results

HISTOLOGY AND SPECIMEN RADIOGRAPHY OF RHEUMATOID ARTHRITIS SYNOVIAL SPECIMENS
The specimens of rheumatoid synovium showed the typical histological features of this condition with villous thickening of the synovial membrane, intimal hyperplasia, and a heavy subintimal chronic inflammatory infiltrate, including numerous lymphocytes, plasma cells, and macrophages. On specimen radiography, it was noted that all rheumatoid synovial specimens contained small radio-opaque fragments, and on histology these were found to represent small fragments of bone derived from the eroded joint; several of these bone fragments were surrounded by osteoclasts.

CHARACTERISATION OF CELLS ISOLATED FROM PERIPHERAL BLOOD AND SYNOVIUM IN 24 h CULTURES

Adherent cells isolated from the peripheral blood of rheumatoid arthritis patients were characterised as monocytes on the basis that, after being incubated alone on coverslips for 24 hours, they were entirely negative for osteoclast markers (that is, TRAP, VNR, and CTR)^{29 31 34}; they strongly expressed CD11b and CD14 monocyte/macrophage antigens, which are known not to be expressed by osteoclasts.³² In addition, after 24 hours incubation on bone slices, no evidence of lacunar resorption was found on scanning electron microscopy.^{35 36} The above cytochemical, immunocytochemical, calcitonin receptor, and functional characteristics were seen when these isolated cells were cultured both in the presence or absence of UMR 106 cells, 1,25(OH)₂D₃, and M-CSF.

Cells isolated from the synovium of rheumatoid arthritis patients cultured for 24 hours on glass coverslips, both in the presence and absence of UMR 106 cells, 1,25(OH)₂D₃, and M-CSF, were also strongly CD11b and CD14 positive. Similarly, they were also entirely negative for the osteoclast markers, TRAP and VNR, and they did not produce lacunar resorption on bone slices.

CYTOCHEMICAL AND IMMUNOPHENOTYPIC CHARACTERISATION OF CELLS ISOLATED FROM PERIPHERAL BLOOD AND SYNOVIUM IN 7 d CULTURES

After seven days incubation on glass coverslips, co-cultures of rheumatoid arthritis monocytes and UMR 106 cells, in the presence of 1,25(OH)₂D₃ and M-CSF, contained numerous multinucleated cells which were positive for TRAP and VNR. In the absence of either UMR 106 cells, M-CSF, or 1,25(OH)₂D₃, only a few TRAP positive multinucleated cells and no VNR positive multinucleated cells were seen in some cultures. Scattered TRAP and VNR positive mononuclear cells were seen in all co-cultures after seven days incubation. Numerous CD11b and CD14 positive mononuclear cells and occasional multinucleated cells positive for these antigens were found

Figure 3 Rheumatoid monocyte/UMR 106 co-culture after 14 d showing the presence of calcitonin receptors on multinucleated cells by autoradiography using ¹²⁵I labelled calcitonin ($\times 400$).

Figure 4 (A) Lacunar resorption pits on human cortical bone slice seen in rheumatoid synovial macrophage/UMR 106 co-culture after 14 d incubation (black bar = 100 μ m). (B) Higher power of one of the complex resorption pits (black bar = 100 μ m).

in seven day cultures both in the presence and absence of UMR 106 cells.

Rheumatoid synovial macrophages cultured for seven days with UMR 106 cells, in the presence of $1,25(\text{OH})_2\text{D}_3$ and M-CSF, also contained numerous TRAP positive (fig 1) and VNR positive mononuclear and multinucleated cells. When incubated in the absence of UMR 106 cells, $1,25(\text{OH})_2\text{D}_3$ or M-CSF, all these cultures were found to contain mainly CD11b and CD14 positive mononuclear cells; however, cultures from two cases, incubated in the absence of the above factors, contained occasional TRAP and VNR positive multinucleated cells. However, scattered TRAP and VNR positive mononuclear cells were seen in all co-cultures after seven days incubation.

LACUNAR BONE RESORPTION AND CALCITONIN RECEPTOR EXPRESSION IN 14 d CO-CULTURES OF RHEUMATOID MONOCYTES AND MACROPHAGES WITH OSTEOBLAST-LIKE CELLS

After 14 days in culture, numerous resorption pits were seen on bone slices upon which rheumatoid monocytes had been co-cultured with

UMR 106 cells in the presence of $1,25(\text{OH})_2\text{D}_3$, M-CSF, and dexamethasone. Lacunar excavation was considerable, amounting to over 500 resorption pits (approximately 20% of the bone slice area), in some cases (fig 2). In all cases, over 50 resorption pits were seen on each bone slice when rheumatoid monocytes were cultured under these conditions. This extensive lacunar resorption was not seen when rheumatoid monocytes were cultured for 14 days in the absence of UMR 106 cells, or when $1,25(\text{OH})_2\text{D}_3$ or M-CSF was omitted from monocyte-UMR 106 co-cultures. Although dexamethasone was not an absolute requirement for lacunar bone resorption, its addition to rheumatoid monocyte-UMR 106 co-cultures (with added $1,25(\text{OH})_2\text{D}_3$ and M-CSF) considerably increased the extent of the lacunar resorption seen.

The presence of calcitonin receptors was also determined by autoradiography in 14 day monocyte cultures, where it was found that CTR positive multinucleated cells were present only in co-cultures of rheumatoid monocytes and UMR 106 cells which had been incubated in the presence of $1,25(\text{OH})_2\text{D}_3$ and M-CSF (fig 3). When monocytes were incubated alone on glass coverslips, or with UMR 106 cells alone, or with UMR 106 in the absence of $1,25(\text{OH})_2\text{D}_3$ or M-CSF, CTR positive cells were not seen.

Fourteen day co-cultures of rheumatoid arthritis synovial macrophages with UMR 106 cells, when incubated with $1,25(\text{OH})_2\text{D}_3$, M-CSF, and dexamethasone, also showed evidence of extensive lacunar resorption on all bone slices, over 200 resorption pits being noted in some cases (fig 4). In the absence of UMR 106 cells, 14 day rheumatoid arthritis macrophage cultures on bone slices, even when incubated in the presence of $1,25(\text{OH})_2\text{D}_3$ and M-CSF, showed no evidence of lacunar resorption. Lacunar resorption was also not seen in rheumatoid macrophage-UMR 106 co-cultures when $1,25(\text{OH})_2\text{D}_3$ or M-CSF was omitted from the culture medium.

Discussion

This is the first report of human osteoclast formation and bone resorption by mononuclear phagocytes isolated from patients with rheumatoid arthritis. It indicates that mononuclear phagocytes, either present in the inflamed synovial membrane or recruited to the synovium from the circulation, may directly contribute to the osteolysis of rheumatoid arthritis by themselves differentiating into multinucleated cells which show all the cytochemical and functional features of osteoclasts. This study has also characterised the cellular and humoral conditions required for this to occur, showing that osteoblastic cells, $1,25(\text{OH})_2\text{D}_3$, and M-CSF are essential for osteoclast formation from rheumatoid monocytes and macrophages.

Cells isolated from the peripheral blood and synovial membrane were characterised as monocytes and macrophages on the basis that they did not express the osteoclast markers of

TRAP,²⁹ VNR,³¹ and CTR,³⁴ and did not possess the ability to form resorption lacunae after 24 hours in culture, a functional feature which is characteristic of osteoclasts^{35,36}; these cells, however, did express CD11b and CD14 mononuclear phagocyte antigens which are known not to be expressed by osteoclasts.³² Although cells expressing these antigens persisted in all the long term monocyte/macrophage-UMR 106 co-cultures examined, it was noted that multinucleated cells which expressed the above osteoclast phenotypic characteristics, including that of lacunar resorption, formed only when rheumatoid monocytes or macrophages were co-cultured with UMR 106 cells in the presence of 1,25(OH)₂D₃ and M-CSF.

Previous studies have shown that mouse monocytes and macrophages are capable of osteoclast differentiation when co-cultured with UMR 106 cells or other bone derived stromal cell types in the presence of 1,25(OH)₂D₃ alone.^{19,20,21-22,37} The present study shows that the above factors are also essential for the in vitro formation of human osteoclasts from rheumatoid arthritis monocytes and macrophages, and that M-CSF is an additional essential cofactor. This requirement for human M-CSF is not surprising as it has previously been shown that rodent M-CSF (which would be secreted by UMR 106 cells) does not bind to the human M-CSF receptor (which would be present on the mononuclear phagocytes isolated from the circulation or synovium of rheumatoid arthritis patients).³⁸ M-CSF is known to be necessary for the proliferation and differentiation of haematopoietic osteoclast progenitors,^{25,39} and an abnormality in the gene for M-CSF has been found to be the cause of the deficiency in osteoclast and bone macrophage numbers in osteopetrotic *op/op* mice.⁴⁰⁻⁴² Synovial macrophages are themselves known to produce 1,25(OH)₂D₃,⁴³ and M-CSF is produced by both bone cells and cells found within the inflamed synovium.^{24,44,45-47} The synovial membrane is known to be a source of other cytokines and growth factors which influence osteoclast differentiation.⁴⁸⁻⁵¹ One of these cytokines is interleukin (IL)-6, receptors for which have been shown to be induced on osteoblastic cells by dexamethasone²⁴; this may explain the marked increase in bone resorption we observed when dexamethasone was added to monocyte/macrophage-UMR 106 co-cultures. IL-6 and soluble IL-6 receptors in the synovial fluid of rheumatoid patients have also recently been shown to be associated with the formation of osteoclast-like cells.⁵¹

Analysis of the immunophenotype of synovial lining cells and subintimal macrophages in the synovial membrane has shown that these cells strongly express monocyte/macrophage markers such as HLA-DR, Fc, and C₃ receptors, as well as a wide range of leucocyte/macrophage antigens, including CD11b and CD14.⁶⁻⁸ In rheumatoid arthritis, when the synovial intima becomes hyperplastic, over 90% of the synovial lining cells are commonly of macrophage phenotype, and

there is an increase in macrophages in the subintima.⁹ Cellular kinetic and immunophenotypic studies have shown that synovial macrophages are derived from circulating monocytes which pass through tall endothelial cells of postcapillary venules before migrating to their tissue location in the intima or subintima.^{4,5,10} These synovial macrophages differentially express certain activation markers which are associated with their ability to carry out a number of complex functions.⁵² In rheumatoid arthritis, circulating monocytes themselves have been reported to show enhanced metabolic and phagocytic activity and to express surface markers consistent with macrophage activation.^{53,54} Monocytes are known to be chemotactically attracted to constituents of the bone matrix,⁵⁵ and cytochemical and ultrastructural studies have shown that macrophage-like mononuclear cells appear at resorption surfaces before mononuclear cells which express osteoclast characteristics.⁵⁶

Synovial macrophages are believed to play a crucial role in joint destruction in rheumatoid arthritis. They are known to release numerous cytokines, principally IL-1 and tumour necrosis factor α ,^{15,16} which act on osteoblasts to stimulate osteoclastic bone resorption indirectly; in addition, they are known to release or to promote the release of prostaglandins and tissue proteases which may play a role in bone lysis.^{14,16,17} However, mature tissue macrophages, even in the presence of these factors, are incapable of causing lacunar resorption of a mineralised substrate.³⁶ This is also the case for rheumatoid synovial macrophages, as confirmed in this study.⁵⁷ However, our findings do indicate that synovial macrophages, like monocytes, are capable of altering their phenotype to that of osteoclasts when cultured under specific cellular and humoral conditions. In the same manner as osteoclast precursors derived from haematopoietic tissues, these mononuclear phagocytes acquire osteoclastic features and lose their macrophage phenotypic features respectively in the process of osteoclast differentiation. Monocyte-osteoclast and macrophage-osteoclast differentiation does not appear to be particular to rheumatoid arthritis, as we have shown that in both man and experimental animals peripheral blood mononuclear cells and tissue macrophages are capable of osteoclast differentiation when cultured with osteoblast-like cells under similar culture conditions.^{20,37,59}

Monocytes and macrophages do not represent homogeneous cell populations but include a fraction of morphologically indistinguishable immature cells capable of further division.⁶⁰ In the context of bone resorption in rheumatoid arthritis, it is possible that these immature mononuclear phagocytes, either as monocytes recruited into the synovium or as synovial macrophages, are capable—when placed in the cellular and humoral microenvironment of bone (that is, when they come in contact with bone lined by osteoblasts in the presence of 1,25(OH)₂D₃ and M-CSF)—of proliferation and differentiation into bone resorbing osteoclasts, the tissue

specific mononuclear phagocyte of bone. The essential cellular and humoral conditions for human osteoclast formation from monocytes and macrophages are present in the in vitro co-culture system described in this study. It should provide a useful model for the analysis of the role that synovial macrophage-osteoclast differentiation plays in the bone destruction associated with rheumatoid arthritis and other arthritic diseases.

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