

EXTENDED REPORT

Synovial macrophage-osteoclast differentiation in inflammatory arthritis

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Background: Pathological bone resorption (marginal erosions and juxta-articular osteoporosis) by osteoclasts commonly occurs in rheumatoid arthritis (RA).

Objectives: To define the nature of the mononuclear precursor cells from which osteoclasts are formed in inflamed synovial tissues and to determine the cellular and humoral factors which influence osteoclast differentiation.

Method: Macrophage (CD14+), non-macrophage (CD14–), and unsorted (CD14+/CD14–) synovial cell populations from RA and inflammatory/non-inflammatory osteoarthritis (OA) synovium were cultured in the presence of receptor activator for nuclear factor κ B ligand (RANKL) and monocyte-colony stimulating factor (M-CSF; in the presence/absence of prostaglandin E₂ (PGE₂), interleukin 1 β (IL1 β), tumour necrosis factor α (TNF α), and IL6). Osteoclast differentiation was assessed by expression of tartrate resistant acid phosphatase (TRAP), vitronectin receptor (VNR), and lacunar resorption.

Results: TRAP+ and VNR+ multinucleated cells capable of lacunar resorption were only formed in cultures of CD14+ containing synovial cell populations (that is, CD14+ and CD14+/CD14– cells). No difference in the extent of osteoclast formation was noted in cultures of CD14+ cells isolated from RA, inflammatory OA, and non-inflammatory OA synovium. However, more TRAP+/VNR+ cells and more lacunar resorption was noted in CD14+/CD14– cells from RA and inflammatory OA synovial tissues. The addition of PGE₂, IL1 β , TNF α , and IL6 did not increase RANKL/M-CSF-induced osteoclast formation and lacunar resorption of both CD14+/CD14– and CD14+ synovial cell populations.

Conclusions: Osteoclast precursors in synovial tissues are CD14+ monocyte/macrophages. The increase in osteoclast formation in cultures of CD14+/CD14– compared with CD14+ synovial cells in RA and inflammatory OA points to a role for CD14– cells in promoting osteoclast differentiation and bone resorption in inflamed synovial tissues by a mechanism which does not involve a direct effect of proinflammatory cytokines/prostaglandins on RANKL-induced macrophage-osteoclast differentiation.

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Rheumatoid arthritis (RA) is an inflammatory joint disease characterised by abnormal synovial proliferation and destruction of articular cartilage and bone. The degree of joint damage in RA correlates with the number of synovial macrophages present in the thickened synovial lining and the subintima, particularly at the articular margins.¹ Several studies have shown that RA synovial cells release proinflammatory prostaglandins and cytokines, such as interleukin (IL) 1, tumour necrosis factor alpha (TNF α), and IL6, all of which are known to promote osteoclastic bone resorption.^{2–4}

Osteoclasts are multinucleated cells which are specialised to carry out lacunar bone resorption.⁵ Mononuclear osteoclast precursors are generated in the bone marrow and circulate in the monocyte fraction.⁶ We have previously shown that osteoclast precursors are found in both the peripheral blood and synovial tissues of patients with RA.^{7,8} Osteoclast precursors express the receptor activator for nuclear factor κ B (RANK)⁹ and differentiate into fully functional osteoclasts in the presence of macrophage-colony stimulating factor (M-CSF) and RANK ligand (RANKL).^{10,11} Binding of RANKL with RANK is inhibited by osteoprotegerin (OPG) a soluble receptor for RANKL.¹² RANK is expressed by T and B lymphocytes and dendritic cells. RANKL and OPG are expressed by osteoblasts, fibroblasts, and inflammatory cells, including T lymphocytes.¹³ Expression of these molecules has been identified in RA and other forms of autoimmune arthritis where these cell types are found.^{14–19} The expression of OPG and RANKL is known to be modulated by prostaglandins and

cytokines including; IL1, TNF α , and IL6.^{20,21} Levels of these cytokines are raised in the synovial fluid of patients with RA.²² Treatment with specific inhibitors/antibodies directed against these inflammatory factors has been shown to be therapeutic in recent trials.^{23–25}

In this study we sought to examine the cellular and humoral mechanisms whereby osteoclast formation contributes to pathological bone destruction in RA and other inflammatory joint diseases. We have defined the nature of the mononuclear osteoclast precursor which is present in synovial tissues. We have also assessed whether the inflammatory microenvironment in synovial tissues influences osteoclastogenesis by comparing osteoclast formation in RA and both inflammatory and non-inflammatory osteoarthritis (OA). In addition, we have also determined the role of non-macrophage synovial cells on osteoclast formation in inflammatory and non-inflammatory joint disease and examined whether proinflammatory humoral factors stimulate RANKL/M-CSF-induced osteoclast differentiation in RA and OA.

Abbreviations: Dex, dexamethasone; FCS, fetal calf serum; IL, interleukin; M-CSF, macrophage-colony stimulating factor; α MEM, alpha minimum essential medium; OA, osteoarthritis; OPG, osteoprotegerin; PGE₂, prostaglandin E₂; RA, rheumatoid arthritis; RANK, receptor activator for nuclear factor κ B; RANKL, RANK ligand; sIL6R, soluble interleukin 6 receptor; TNF α , tumour necrosis factor α ; TRAP, tartrate resistant acid phosphatase; VNR, vitronectin receptor

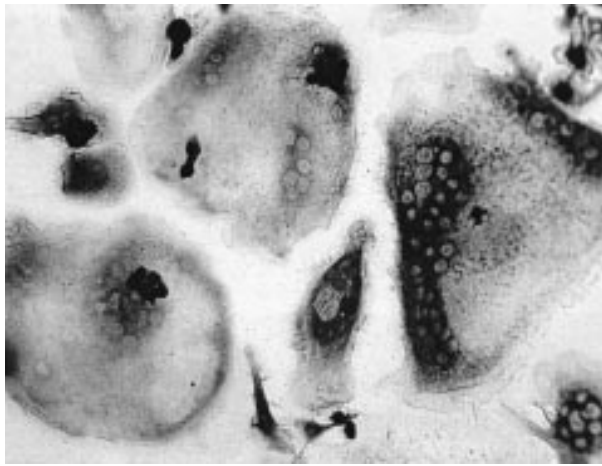


Figure 1 Seven day culture of RA CD14+/CD14- synovial cells incubated with M-CSF (25 ng/ml), RANKL (30 ng/ml), and Dex (10^{-8} M) showing numerous multinucleated cells positive for TRAP. Original magnification $\times 200$.

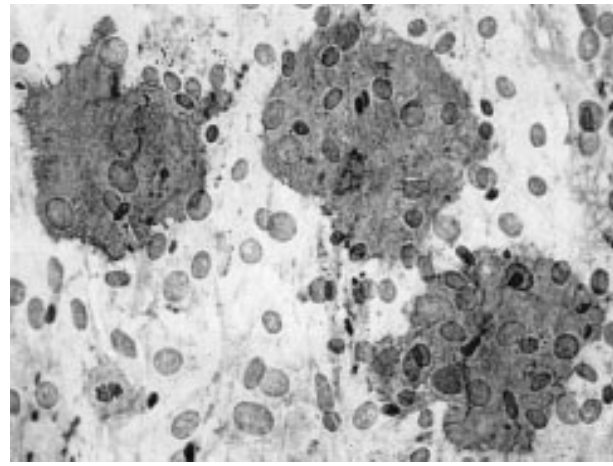


Figure 2 Seven day culture of RA CD14+/CD14- synovial cells incubated with M-CSF (25 ng/ml), RANKL (30 ng/ml), and Dex (10^{-8} M) showing numerous multinucleated cells positive for VNR. Original magnification $\times 200$.

MATERIALS AND METHODS

Reagents

Incubations were performed in alpha minimal essential medium (α MEM; Gibco, UK) supplemented with 10 mM L-glutamine, 100 IU/ml penicillin, 10 μ g/ml streptomycin (Gibco, UK) and 10% heat inactivated fetal calf serum (FCS) (Gibco, UK). Collagenase type 1, dexamethasone (Dex), prostaglandin E₂ (PGE₂) and indometacin were purchased from Sigma Chemicals (UK). Amgen Inc (Thousand Oaks, CA USA) kindly provided the soluble RANKL. Recombinant human M-CSF, IL1 β , TNF α , IL6, soluble IL6 receptor (sIL6R) and corresponding antibodies to these cytokines were purchased from R&D Systems (UK).

RA and OA cases studied and isolation of cells from synovial tissues

During total hip, knee, and elbow replacement surgery, synovial tissue was taken from 11 patients with seropositive RA (eight female, three male, average age 70.5 years) and 18 patients with OA (14 female, four male, average age 72.5 years). The diagnosis of RA and primary OA was made on the basis of clinical examination and radiological investigations as well as by relevant serological and other investigations to exclude other causes of arthritis. Patients with OA were divided into those who had histologically non-inflammatory OA synovium (six female, two male, average age 71.8 years) and those who had inflammatory OA synovium (eight female, two male, average age 73.2 years) on the basis of the extent of the subintimal inflammatory infiltrate.²⁶⁻²⁸ Patients who were found to have inflammatory or non-inflammatory OA presented identically with the usual clinical and radiological features of primary OA.

Synovial tissue specimens were washed thoroughly with phosphate buffered saline before being cut into small segments and digested in α MEM containing 1 mg/ml collagenase type 1 for one hour at 37°C; this was followed by a further 45 minutes' incubation in Versine (Gibco, UK). The digested tissue was filtered through a 70 μ m cell strainer (Falcon, UK) and the filtrate centrifuged at 800 *g* for 10 minutes. The cell pellet was resuspended in running buffer (phosphate buffered saline pH 7.2, 2 mM EDTA, 0.5% bovine serum albumin) and filtered again through a 30 μ m pre-separation filter (Miltenyi Biotec, Germany). The cells were counted in a haemocytometer after lysis of red blood cells using a 5% vol/vol acetic acid solution.

Sorting of CD14+ and CD14- cell populations from RA and OA synovial tissues

Cell sorting was performed using the MidiMACS separation kit (Miltenyi Biotec Ltd, UK). Cells derived from RA and OA synovium were incubated with MACS CD14 MicroBeads (Miltenyi Biotec) for 20 minutes at 4°C. The cells were washed in running buffer and passed through a MACS magnetic cell separator. The cells were sorted into a CD14+ cell fraction and a CD14- cell fraction. The cells were washed and resuspended in α MEM/FCS then counted in a haemocytometer after lysis of red blood cells as described above.

Culture of sorted (CD14+ or CD14-) and unsorted (CD14+/CD14-) cell populations with RANKL and M-CSF

Separated populations of CD14+ and CD14- cells and unsorted CD14+/CD14- cells, were each added (1×10^5 cells/well) to 96 well tissue culture plates containing glass coverslips and dentine slices prepared as previously described.^{7,8} After two hours' incubation, dentine slices and coverslips were removed from the wells, washed vigorously in α MEM/FCS to remove non-adherent cells and then placed in 16 mm wells of a 24 well tissue culture plate containing 1 ml of α MEM/FCS supplemented with RANKL (30 ng/ml), M-CSF (25 ng/ml), and Dex (10^{-8} M). All cultures were incubated for 1, 7, and 14 days and the culture medium containing these factors was replenished every 3-4 days. Control cultures consisted of synovial cells incubated in the absence of RANKL and/or M-CSF.

Cytochemical assessment of osteoclast formation

Synovial cell cultures incubated for 24 hours and 7 days on glass coverslips were examined for the expression of the osteoclast associated marker tartrate resistant acid phosphatase (TRAP) using a commercially available kit: 386-A (Sigma, UK).²⁹ Cell cultures on coverslips were also stained immunohistochemically by an indirect immunoperoxidase technique using 23C6 (Serotec, UK), a monoclonal antibody to the vitronectin receptor (VNR), an osteoclast associated antigen,³⁰ and JML-H14 a monoclonal antibody to the monocyte/macrophage associated antigen CD14,³¹ which is not expressed by osteoclasts.³²

Functional assessment of the extent of lacunar resorption

The extent of lacunar resorption was measured after 24 hours and 14 days' incubation of synovial cell cultures on dentine

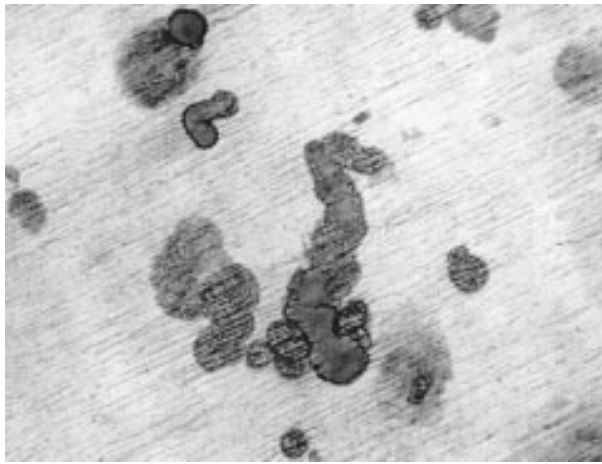


Figure 3 Fourteen day culture of RA CD14+/CD14- synovial cells incubated on dentine slices in the presence of M-CSF (25 ng/ml), RANKL (30 ng/ml), and Dex (10^{-8} M). The cells have been removed and the dentine slices stained with toluidine blue to reveal the formation of numerous well defined lacunar resorption pits. Original magnification $\times 20$.

slices. After incubation the slices were left overnight in 1M ammonium hydroxide, then washed in distilled water and cleaned by ultrasonication. Resorption pits were examined by light microscopy after staining with toluidine blue. The mean percentage area resorbed on each dentine slice was then measured using Adobe PhotoShop 5.5 software for image analysis.

Effect of PGE₂, IL1, TNF α , and IL6/sIL6R on osteoclast formation and lacunar resorption

CD14+/CD14- and CD14+ cell cultures were set up on glass coverslips and dentine slices in the presence of RANKL (30 ng/ml), M-CSF (25 ng/ml), and Dex (10^{-8} M) with the following proinflammatory factors: PGE₂ (10^{-4} – 10^{-8} M), IL1 β (0.01–5 ng/ml), TNF α (0.1–50 ng/ml), IL6 (10–500 ng/ml) with sIL6R (10–500 ng/ml). Indometacin (10^{-6} M), and corresponding antibodies to the above cytokines (10 μ g/ml) were also added to the above cell cultures. All cultures were incubated for 1, 7, and 14 days. The culture medium containing these factors was replenished every 3–4 days. Control cultures consisted of incubation of synovial cells in the absence of RANKL and/or M-CSF.

TUNEL staining of cultured cells

CD14+/CD14- cell cultures on coverslips were removed after 24 hours and 7 days' incubation and cells were air dried and fixed overnight in 1% paraformaldehyde. TUNEL staining to identify apoptotic cells in these cultures was performed using the ApopTag kit (Intergen Company, UK). Cultures treated with added factors were compared with cell cultures to which no PGE₂, cytokines, indometacin, or specific cytokine antibodies had been added.

Statistical analysis

Each experiment was carried out in triplicate. Statistical analysis on measurements of mean percentage area resorbed was performed with the Mann Whitney U test. Values $p < 0.05$ were considered significant.

RESULTS

Osteoclast differentiation in CD14+/CD14- and CD14+ RA and OA synovial cell populations

Sorted (CD14+ or CD14-) and unsorted CD14+/CD14- synovial cells cultured in the presence of RANKL, M-CSF, and Dex, were assessed for cytochemical (TRAP/VNR) and functional (lacunar resorption) evidence of osteoclast differentiation after 1, 7 and 14 days' incubation. TRAP+ (fig 1) and VNR+ (fig 2) cells were only formed in sorted CD14+ and unsorted CD14+/CD14- cells derived from RA and OA synovial tissues. They did not form in CD14- cell cultures. In 14 day cultures on dentine slices, extensive lacunar resorption was only seen in cultures of sorted CD14+ and unsorted CD14+/CD14- cells (fig 3). These findings indicate that mononuclear osteoclast precursors in synovial tissues express the monocyte/macrophage marker CD14.

Osteoclast formation, as assessed by the presence of TRAP+ and VNR+ cells in 7 day cultures and the extent of lacunar resorption in 14 day cultures, was significantly increased in cultures of CD14+/CD14- synovial cells from RA ($p = 0.0001$) and inflammatory OA ($p < 0.0001$) compared with non-inflammatory OA (fig 4). In contrast, there was no difference in the extent of osteoclast formation in cultures of CD14+ cells isolated from RA, inflammatory OA, and non-inflammatory OA synovium. Osteoclast formation and lacunar resorption was significantly increased in cultures of CD14+/CD14- synovial cells compared with CD14+ synovial cells from RA ($p = 0.01$) and inflammatory OA ($p = 0.02$) synovium. Conversely, in non-inflammatory OA, osteoclast formation and lacunar resorption was significantly less in cultures of

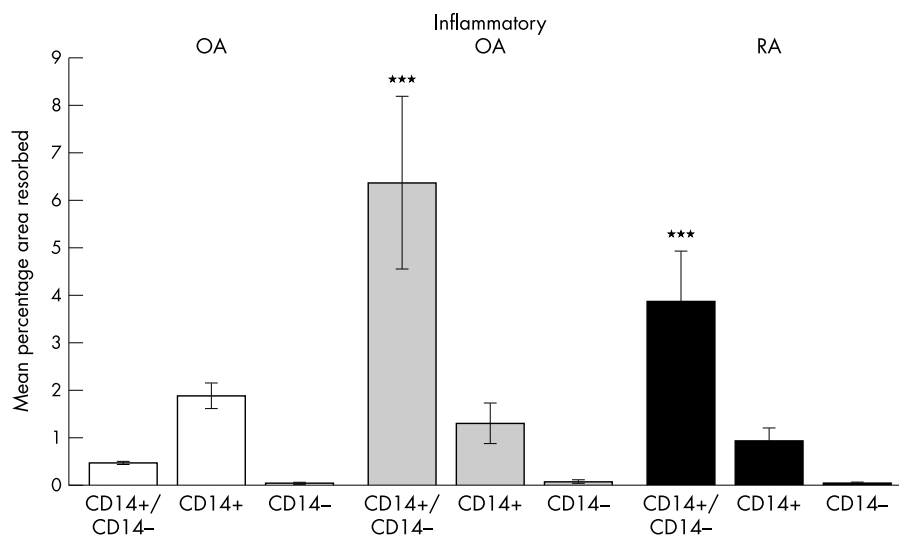


Figure 4 Mean percentage area resorption of OA, inflammatory OA and RA CD14+, CD14+/CD14-, and CD14- synovial cells cultured in the presence of M-CSF (25 ng/ml), RANKL (30 ng/ml), and Dex (10^{-8} M). *** $p < 0.0005$ relative to CD14+/CD14- OA (control) cell cultures.

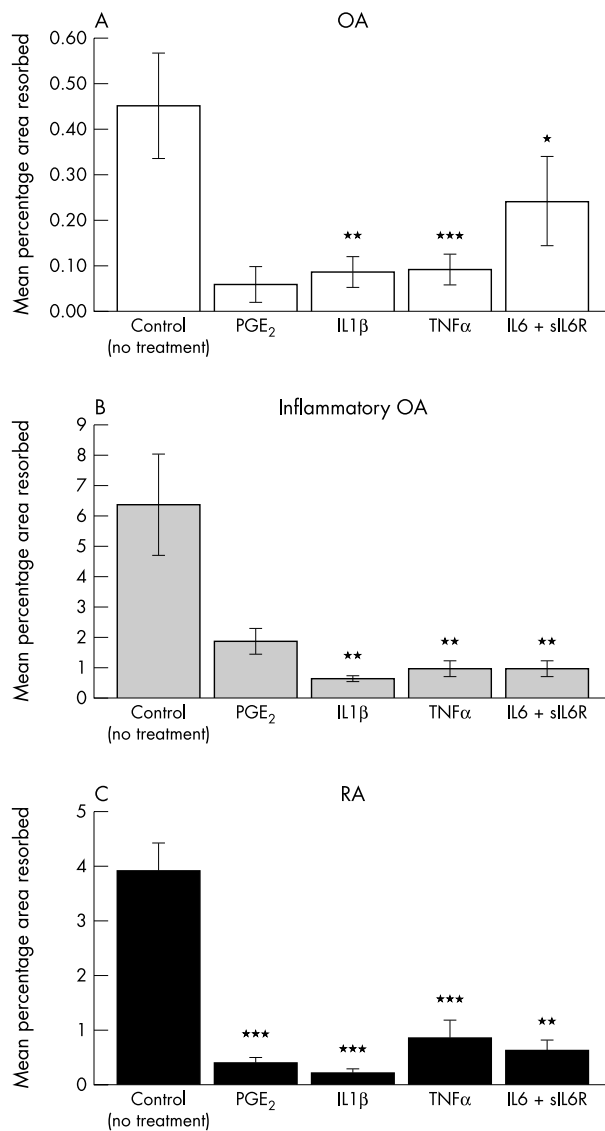


Figure 5 Mean percentage area resorption of CD14+/CD14- synovial cells cultured with M-CSF (25 ng/ml), RANKL (30 ng/ml), and Dex (10^{-8} M) in the presence/absence of PGE₂ (10^{-6} M), IL1β (1 ng/ml), TNFα (10 ng/ml), and IL6 (100 ng/ml) with sIL6R (100 ng/ml) in (A) non-inflammatory OA; (B) inflammatory OA; (C) RA. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$ relative to untreated controls.

CD14+/CD14- cells than in sorted CD14+ cells ($p = 0.02$). This finding points to a role for CD14- cells in promoting osteoclast formation and bone resorption in inflamed synovial tissues.

Effect of PGE₂, IL1β, TNFα, and IL6 on osteoclast differentiation

To determine the effect of prostaglandins and cytokines on osteoclast formation in inflammatory and non-inflammatory synovial tissues, these factors were added to 7 and 14 day cultures of sorted (CD14+) and unsorted (CD14+/CD14-) cultures of RA and OA synovial cells. The addition of these proinflammatory humoral factors to 7 and 14 day cultures of both sorted and unsorted synovial cells reduced the number of TRAP+ and VNR+ multinucleated cells and the extent of lacunar resorption (fig 5). IL1β significantly inhibited lacunar resorption in cultures of synovial cells derived from RA ($p < 0.0001$), inflammatory OA ($p = 0.002$), and non-inflammatory OA ($p = 0.001$) joints. TNFα inhibited lacunar resorption in synovial cell cultures from RA ($p = 0.0004$), inflammatory OA ($p = 0.003$), and non-inflammatory OA

($p < 0.0001$) joints. IL6 inhibited lacunar resorption in synovial cell cultures isolated from RA ($p = 0.002$), inflammatory OA ($p = 0.006$), and non-inflammatory OA ($p = 0.022$) joints. PGE₂ also inhibited lacunar resorption in all synovial cell cultures, although this inhibition was only significant in synovial cell cultures from RA joints ($p < 0.0001$). The addition of indometacin, anti-IL1β, and anti-IL6 antibodies to unsorted CD14+/CD14- RA, inflammatory OA, and non-inflammatory OA synovial cell cultures had no significant effect on osteoclast differentiation or lacunar resorption. The addition of anti-TNFα to cultures of unsorted RA synovial cells resulted in a small but significant increase in lacunar resorption.

To determine if the inhibition of osteoclast formation in the presence of the above proinflammatory humoral factors was due to increased apoptosis of osteoclast precursors, we carried out TUNEL staining of 24 hour and 7 day cultures of unsorted RA and OA synovial cells. There was no difference in the number of TUNEL positive cells in 24 hour and 7 day RA and OA synovial cell cultures incubated in the presence or absence of PGE₂, IL1β, TNFα, IL6/sIL6R, indometacin, or corresponding anticytokine antibodies. In all cases, 7 day synovial cell cultures contained <1% TUNEL positive cells.

DISCUSSION

Synovial macrophages are present in both the synovial intima and subintima and are abundant in joints affected by RA and OA.¹³ Analysis of the immunophenotype of synovial lining cells and subintimal macrophages in RA and OA has shown that these cells express a wide range of monocyte/macrophage markers including HLA-DR, CD11b, and CD14.³⁴ Synovial macrophages secrete numerous cytokines, principally IL1 and TNFα, which act on osteoblasts to stimulate osteoclastic bone resorption indirectly. In addition, synovial macrophages release prostaglandins and tissue proteases which may enhance osteoclastic bone resorption.³³

We have previously shown that cells isolated from the monocyte fraction of peripheral blood and macrophage-like cells isolated from the synovial membrane of patients with RA are capable of osteoclast formation when cultured in the presence of M-CSF and RANKL or RANKL-expressing bone stromal cells.^{7,8} In this study we have shown, through culture of sorted and unsorted synovial cell populations, that mononuclear osteoclast precursors in RA and OA synovial tissues express the monocyte/macrophage marker CD14. Osteoclast differentiation only occurred in synovial cell populations which contained either CD14+ cells alone or CD14+/CD14- cells, and was not seen in cultures of CD14- cells alone. No difference was noted in the extent of osteoclast formation from CD14+ mononuclear phagocyte precursors in RA and OA (inflammatory or non-inflammatory) synovium. However, there was a striking increase in osteoclast formation and lacunar resorption in cultures of unsorted (CD14+/CD14-) synovial cells from RA and inflammatory OA compared with non-inflammatory OA joints. This finding indicates that CD14- synovial cells isolated from inflamed synovial tissues markedly promote osteoclast formation. The identity of the CD14- cells promoting macrophage-osteoclast formation is unknown, although both stromal (for example, synovial fibroblasts) and inflammatory cell (for example, T lymphocytes) are likely candidates for this role. Cultured synovial fibroblasts have been shown to induce osteoclast formation,^{17,35} and are capable of producing M-CSF,³⁶ an absolute requirement for osteoclast formation. Synovial fibroblasts cultured from patients with RA have recently been shown to express mRNA and protein for RANKL.¹⁷ Fibroblasts isolated from non-synovial tissues have also been shown to express RANKL and to be capable of supporting osteoclast formation of peripheral blood mononuclear cells in mice.³⁷ Activated human T cells have also been shown to express RANKL and to induce osteoclastogenesis from human monocytes.¹⁶

The synovial membrane is known to contain a number of proinflammatory humoral factors which influence osteoclast formation and bone resorption. PGE₂, IL1 α , and TNF α have been shown to promote osteoclast formation in mouse marrow cultures, predominantly through an increase in RANKL mRNA expression by bone stromal cells.¹³ IL1 has been shown to induce multinucleation and bone resorbing activity of osteoclasts in the absence of osteoblasts.³⁸ In addition, TNF α has recently been reported to stimulate osteoclast formation in the presence of permissive levels of RANKL, and TNF α and IL6 have been shown to induce mouse and human osteoclast formation by a RANKL independent mechanism.^{39–42} We found that the addition of PGE₂, IL1, and TNF α inhibited (RANKL-induced) osteoclast formation of sorted CD14+ and unsorted CD14+/CD14– cells isolated from RA and inflammatory/non-inflammatory OA synovial tissues. PGE₂ is known to inhibit human osteoclast formation from marrow and circulating precursors *in vitro*,^{43–44} but the mechanism whereby macrophage-osteoclast differentiation is inhibited in the arthritic synovium is unknown. Many of the above proinflammatory factors stimulate osteoclast formation and resorption by acting via osteoblasts which are absent from our cultures; this would also explain the lack of effect by the respective anticytokine antibodies. IL1 and TNF α are known to stimulate the formation of OPG in human osteoblast-like cells,^{45–47} and, possibly, these cytokines may also be inducing production of OPG by synovial macrophages that are present in our cultures.¹³ TNF α and IL1 have been shown to stimulate soluble OPG production in synovial fluid mononuclear cells and synovial fibroblasts in patients with RA.⁴⁸ These cytokine factors may also operate through transducing signalling pathways that are shared with the RANKL signalling pathway, resulting in competitive inhibition of RANKL mediated osteoclast formation. IL1 and TNF α promote translocation of NF κ B to the cell nucleus where it binds the promoter region of a variety of genes. Activation of the P55 TNF receptor which leads to cell apoptosis⁴⁹ does not appear to be the cause of this inhibitory effect as these cytokines did not increase TUNEL staining in CD14+ and CD14+/CD14– RA and OA synovial cell cultures.

The results of this study clearly show that osteoclast precursor cells are present in the CD14+ macrophage population of synovial cells and that non-macrophage cell populations, particularly in the inflamed synovium, can influence the extent of macrophage-osteoclast differentiation. One intriguing aspect of our findings is that CD14– cell promotion of osteoclast formation occurred not only in RA, in which marginal erosions are commonly found, but also in OA, a condition which, in large joints, is not usually associated with periarticular bone resorption even in the presence of florid inflammatory changes in the OA synovium. A rare form of erosive inflammatory OA can occur in the interphalangeal joints of the hand. Possibly, formation of marginal erosions in RA but not OA may be due to a relative increase in synovial cell production of humoral factors which promote RANKL-induced osteoclast formation and bone resorption. The ratio of soluble RANKL to OPG has been shown to be significantly higher in the synovial fluid of patients with RA than in patients with OA.¹⁶ M-CSF is also known to be increased in RA compared with OA synovial fluid.^{50–51} This relative increase in the humoral factors that stimulate osteoclast formation in RA synovial fluid may thus act to increase osteoclastogenesis, resulting in the formation of marginal erosions in RA but not OA.

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REFERENCES

- 1 **Yanni G**, Whelan A, Feighery C, Bresnihan B. Synovial tissue macrophages and joint erosion in rheumatoid arthritis. *Ann Rheum Dis* 1994;53:39–44.
- 2 **Arend WP**, Dayer JM. Cytokines and cytokine inhibitors or antagonists in rheumatoid arthritis. *Arthritis Rheum* 1990;33:305–15.
- 3 **Brennan FM**, Field M, Chu CQ, Feldmann M, Maini RN. Cytokine expression in rheumatoid arthritis. *Br J Rheumatol* 1991;30:76–80.
- 4 **Farahat MN**, Yanni G, Poston R, Panayi GS. Cytokine expression in synovial membranes of patients with rheumatoid arthritis and osteoarthritis. *Ann Rheum Dis* 1993;52:870–5.
- 5 **Athanasou NA**. Cellular biology of bone-resorbing cells. *J Bone Joint Surg Am* 1996;78:1096–112.
- 6 **Fujikawa Y**, Quinn JM, Sabokbar A, McGee JO, Athanasou NA. The human osteoclast precursor circulates in the monocyte fraction. *Endocrinology* 1996;137:4058–60.
- 7 **Fujikawa Y**, Sabokbar A, Neale S, Athanasou NA. Human osteoclast formation and bone resorption by monocytes and synovial macrophages in rheumatoid arthritis. *Ann Rheum Dis* 1996;55:816–22.
- 8 **Itonaga I**, Fujikawa Y, Sabokbar A, Murray DW, Athanasou NA. Rheumatoid arthritis synovial macrophage-osteoclast differentiation is osteoprotegerin ligand-dependent. *J Pathol* 2000;192:97–104.
- 9 **Nakagawa N**, Kinoshita M, Yamaguchi K, Shima N, Yasuda H, Yano K, et al. RANK is the essential signaling receptor for osteoclast differentiation factor in osteoclastogenesis. *Biochem Biophys Res Commun* 1998;253:395–400.
- 10 **Yasuda H**, Shima N, Nakagawa N, Yamaguchi K, Kinoshita M, Mochizuki S, et al. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci USA* 1998;95:3597–602.
- 11 **Lacey DL**, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 1998;93:165–76.
- 12 **Simonet WS**, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 1997;89:309–19.
- 13 **Hofbauer LC**, Khosla S, Dunstan CR, Lacey DL, Boyle WJ, Riggs BL. The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. *J Bone Miner Res* 2000;15:2–12.
- 14 **Kong YY**, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, et al. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* 1999;402:304–9.
- 15 **Romas E**, Bakharevski O, Hards DK, Kartsogiannis V, Quinn JM, Ryan PF, et al. Expression of osteoclast differentiation factor at sites of bone erosion in collagen-induced arthritis. *Arthritis Rheum* 2000;43:821–6.
- 16 **Kotake S**, Udagawa N, Hakoda M, Mogi M, Yano K, Tsuda E, et al. Activated human T cells directly induce osteoclastogenesis from human monocytes: possible role of T cells in bone destruction in rheumatoid arthritis patients. *Arthritis Rheum* 2001;44:1003–12.
- 17 **Takayanagi H**, Iizuka H, Juji T, Nakagawa T, Yamamoto A, Miyazaki T, et al. Involvement of receptor activator of nuclear factor kappaB ligand/osteoclast differentiation factor in osteoclastogenesis from synovial cells in rheumatoid arthritis. *Arthritis Rheum* 2000;43:259–69.
- 18 **Shigeyama Y**, Pap T, Kunzler P, Simmen BR, Gay RE, Gay S. Expression of osteoclast differentiation factor in rheumatoid arthritis. *Arthritis Rheum* 2000;43:2523–30.
- 19 **Haynes DR**, Crotti TN, Loric M, Bain GI, Atkins GJ, Findlay DM. Osteoprotegerin and receptor activator of nuclear factor kappaB ligand (RANKL) regulate osteoclast formation by cells in the human rheumatoid arthritic joint. *Rheumatology (Oxford)* 2001;40:623–30.
- 20 **Brandstrom H**, Bjorkman T, Ljunggren O. Regulation of osteoprotegerin secretion from primary cultures of human bone marrow stromal cells. *Biochem Biophys Res Commun* 2001;280:831–5.
- 21 **Nakashima T**, Kobayashi Y, Yamasaki S, Kawakami A, Eguchi K, Sasaki H, et al. Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-kappaB ligand: modulation of the expression by osteotropic factors and cytokines. *Biochem Biophys Res Commun* 2000;275:768–75.
- 22 **Firestein GS**, Alvaro-Gracia JM, Maki R, Alvaro-Garcia JM. Quantitative analysis of cytokine gene expression in rheumatoid arthritis. *J Immunol* 1990;144:3347–53.
- 23 **Bresnihan B**. Treatment of rheumatoid arthritis with interleukin 1 receptor antagonist. *Ann Rheum Dis* 1999;58(suppl 1):196–8.
- 24 **Elliott MJ**, Maini RN, Feldmann M, Kalden JR, Antoni C, Smolen JS, et al. Randomised double-blind comparison of chimeric monoclonal antibody to tumour necrosis factor alpha (cA2) versus placebo in rheumatoid arthritis. *Lancet* 1994;344:1105–10.
- 25 **Nishimoto N**, Kishimoto T, Yoshizaki K. Anti-interleukin 6 receptor antibody treatment in rheumatic disease. *Ann Rheum Dis* 2000;59(suppl 1):i21–7.
- 26 **Goldenberg DL**, Egan MS, Cohen AS. Inflammatory synovitis in degenerative joint disease. *J Rheumatol* 1982;9:204–9.

- 27 **Revell PA**, Mayston V, Lallor P, Mapp P. The synovial membrane in osteoarthritis: a histological study including the characterisation of the cellular infiltrate present in inflammatory osteoarthritis using monoclonal antibodies. *Ann Rheum Dis* 1988;47:300–7.
- 28 **Lindblad S**, Hedfors E. Arthroscopic and immunohistologic characterization of knee joint synovitis in osteoarthritis. *Arthritis Rheum* 1987;30:1081–8.
- 29 **Minkin C**. Bone acid phosphatase: tartrate-resistant acid phosphatase as a marker of osteoclast function. *Calcif Tissue Int* 1982;34:285–90.
- 30 **Davies J**, Warwick J, Totty N, Philp R, Helfrich M, Horton M. The osteoclast functional antigen, implicated in the regulation of bone resorption, is biochemically related to the vitronectin receptor. *J Cell Biol* 1989;109:1817–26.
- 31 **Knapp W**, Dorken B, Rieber EP, Stein H, Gilks WR, Schmidt RE. *Leukocyte Typing IV: white cell differentiation antigens*: Oxford University Press, New York, 1989.
- 32 **Athanasou NA**, Quinn J. Immunophenotypic differences between osteoclasts and macrophage polykaryons: immunohistological distinction and implications for osteoclast ontogeny and function. *J Clin Pathol* 1990;43:997–1003.
- 33 **Athanasou NA**. Synovial macrophages. *Ann Rheum Dis* 1995;54:392–4.
- 34 **Athanasou NA**, Quinn J, Heryet A, Puddle B, Woods CG, McGee JO. The immunohistology of synovial lining cells in normal and inflamed synovium. *J Pathol* 1988;155:133–42.
- 35 **Takayanagi H**, Oda H, Yamamoto S, Kawaguchi H, Tanaka S, Nishikawa T, *et al*. A new mechanism of bone destruction in rheumatoid arthritis: synovial fibroblasts induce osteoclastogenesis. *Biochem Biophys Res Commun* 1997;240:279–86.
- 36 **Seitz M**, Loetscher P, Fey MF, Tabler A. Constitutive mRNA and protein production of macrophage colony-stimulating factor but not of other cytokines by synovial fibroblasts from rheumatoid arthritis and osteoarthritis patients. *Br J Rheumatol* 1994;33:613–19.
- 37 **Quinn JM**, Horwood NJ, Elliott J, Gillespie MT, Martin TJ. Fibroblastic stromal cells express receptor activator of NF-kappa B ligand and support osteoclast differentiation. *J Bone Miner Res* 2000;15:1459–66.
- 38 **Jimi E**, Nakamura I, Duong LT, Ikebe T, Takahashi N, Rodan GA, *et al*. Interleukin 1 induces multinucleation and bone-resorbing activity of osteoclasts in the absence of osteoblasts/stromal cells. *Exp Cell Res* 1999;247:84–93.
- 39 **Lam J**, Takeshita S, Barker JE, Kanagawa O, Ross FP, Teitelbaum SL. TNF-alpha induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. *J Clin Invest* 2000;106:1481–8.
- 40 **Kobayashi K**, Takahashi N, Jimi E, Udagawa N, Takami M, Kotake S, *et al*. Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction. *J Exp Med* 2000;191:275–86.
- 41 **Kudo O**, Fujikawa Y, Itonaga I, Sabokbar A, Tarisu T, Athanasou NA. Pro-inflammatory cytokine (TNF α /IL-1 α) induction of human osteoclast formation. *J Pathol* (in press).
- 42 **Kudo O**, Sabokbar A, Athanasou NA. Interleukin-6 and interleukin-11 support human osteoclast formation by a RANKL-independent mechanism. *Bone* (in press).
- 43 **Chenu C**, Kurihara N, Mundy GR, Roodman GD. Prostaglandin E2 inhibits formation of osteoclast like cells in long-term human marrow cultures but is not a mediator of the inhibitory effects of transforming growth factor beta. *J Bone Miner Res* 1990;5:677–81.
- 44 **Itonaga I**, Sabokbar A, Neale SD, Athanasou NA. 1,25-Dihydroxyvitamin D(3) and prostaglandin E(2) act directly on circulating human osteoclast precursors. *Biochem Biophys Res Commun* 1999;264:590–5.
- 45 **Hofbauer LC**, Dunstan CR, Spelsberg TC, Riggs BL, Khosla S. Osteoprotegerin production by human osteoblast lineage cells is stimulated by vitamin D, bone morphogenetic protein-2, and cytokines. *Biochem Biophys Res Commun* 1998;250:776–81.
- 46 **Vidal ON**, Sjogren K, Eriksson BI, Ljunggren O, Ohlsson C. Osteoprotegerin mRNA is increased by interleukin-1 alpha in the human osteosarcoma cell line MG-63 and in human osteoblast-like cells. *Biochem Biophys Res Commun* 1998;248:696–700.
- 47 **Brandstrom H**, Jonsson KB, Vidal O, Ljunghall S, Ohlsson C, Ljunggren O. Tumor necrosis factor-alpha and -beta upregulate the levels of osteoprotegerin mRNA in human osteosarcoma MG-63 cells. *Biochem Biophys Res Commun* 1998;248:454–7.
- 48 **Zolkowska M**, Kuroska M RA, Luszczkiewicz G, Dziewczopolski W, Kowalczywski J, Maslinski W. The effects of TNF-alpha, IL-1 beta and IL-15 on the production/expression of OPG and RANKL by synovial fluid mononuclear cells and fibroblast-like synoviocytes isolated from patients with rheumatoid arthritis (RA). *Ann Rheum Dis* 2001;60[suppl 1]:49.
- 49 **Aggarwal BB**. Tumour necrosis factors receptor associated signalling molecules and their role in activation of apoptosis, JNK and NF-kappaB. *Ann Rheum Dis* 2000;59[suppl 1]:i6–16.
- 50 **Moss ST**, Hamilton JA. Proliferation of a subpopulation of human peripheral blood monocytes in the presence of colony stimulating factors may contribute to the inflammatory process in diseases such as rheumatoid arthritis. *Immunobiology* 2000;202:18–25.
- 51 **Smith JB**, Bocchieri MH, Smith JB Jr, Sherbin-Allen L, Abruzzo JL. Colony stimulating factor occurs in both inflammatory and noninflammatory synovial fluids. *Rheumatol Int* 1990;10:131–4.