Synovial fluid macrophages are capable of osteoclast formation and resorption

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Abstract
To determine whether synovial fluid (SF) macrophages isolated from the SF of osteoarthritis (OA), rheumatoid arthritis (RA) and pyrophosphate arthropathy (PPA) joints are capable of osteoclast formation, and to investigate the cellular and humoral factors required for this to occur, SF macrophages (CD14+) were isolated from the knee joint SF from patients with OA, RA and PPA and cultured for up to 14 days with macrophage-colony stimulating factor (M-CSF) and soluble receptor activator for nuclear factor-κB ligand (RANKL) or tumour-necrosis factor-α (TNFα) and interleukin-1α (IL-1α). Osteoclast differentiation was assessed by expression of tartrate-resistant acid phosphatase (TRAP) and vitronectin receptor (VNR), F-actin ring formation and lacunar resorption. Osteoclast formation and lacunar resorption was seen in RANKL-treated cultures of SF macrophages isolated from OA, RA and PPA joints with the largest amount of resorption noted in RA and PPA SF macrophage cultures. In TNFα/IL-1α-treated RA and PPA SF macrophage cultures, osteoclasts capable of lacunar resorption were also formed. Lacunar resorption was more extensive in RANKL than TNFα/IL-1α-treated cultures. These findings indicate that SF macrophages are capable of differentiating into mature osteoclasts capable of lacunar resorption. M-CSF in combination with RANKL or TNFα/IL-1α was required for osteoclast formation. As inflammatory synovial fluids contain an increase in the number of macrophages and an increase in the amounts of RANKL, TNFα and IL-1α, these findings suggest that one means whereby bone erosions may form in rheumatoid or crystal arthritis is by differentiation of synovial fluid macrophages into osteoclasts.

Keywords: synovial fluid; osteoclasts; macrophages; osteoarthritis; rheumatoid arthritis; pyrophosphate

Introduction
Normal synovial fluid (SF) is a dialysate of plasma modified by constituents secreted by cells in joint tissues. Normal SF and SF from uninflamed joints has a high content of hyaluronic acid (HA) and contains relatively few inflammatory cells [1]. In inflammatory joint conditions such as rheumatoid arthritis (RA) and pyrophosphate arthropathy (PPA), which are associated with the formation of subchondral or marginal erosions, the SF contains inflammatory cells, including numerous neutrophil polymorphs, macrophages and lymphocytes [1]. These SF cells may come into direct contact with the cartilage and bone covering the articular surface and may play a significant role in joint destruction.

Pathological bone resorption associated with the formation of marginal erosions in RA is carried out by specialized multinucleated bone-resorbing cells called osteoclasts [2]. Mononuclear phagocyte osteoclast precursors are found in circulating monocyte [3] and tissue macrophage populations (including synovial macrophages) [4,5]. These cells are able to differentiate into multinucleated osteoclasts in the presence of macrophage-colony stimulating factor (M-CSF) and the receptor activator for nuclear factor-κB ligand (RANKL) [6]; the latter is expressed by synovial fibroblasts, T lymphocytes and osteoblasts [7]. Osteoprotegerin (OPG) is a soluble decoy receptor for RANKL that inhibits osteoclast formation and bone resorption [6,8]. RANKL and OPG mRNA expression by bone cells is regulated by numerous cytokines and growth factors [9,10]. An increase in the RANKL:OPG ratio in RA synovial fluid has been noted [11,12]. TNFα and IL-1α are also major components of inflammatory SF and a RANKL-independent mechanism of osteoclast formation has been described, whereby TNFα can substitute for RANKL [13]; this osteoclast formation and resorption activity induced by TNFα is promoted by IL-1α [14].
In this study, our aim has been to determine whether macrophages isolated from SF are capable of osteoclast formation and to examine the cellular and humoral mechanisms by which this occurs. We have also determined whether there are differences in the capacity of synovial macrophages isolated from inflammatory and non-inflammatory arthritic conditions to undergo osteoclast differentiation and carry out lacunar resorption.

Materials and methods

The Oxford Clinical Research Ethics Committee approved this study. SF was aspirated from the knee joints of five OA patients (four males, one female: average age 67 years), six RA patients (four females and two males: average age 46.5 years) and five PPA patients (five males: average age 72). The diagnosis of OA, RA and PPA was made on the basis of clinical, radiological and histological criteria; all RA cases were seropositive for rheumatoid factor. The presence of positively birefringent crystals in PPA SF samples was confirmed by polarization microscopy. A sample of each fluid was also sent for microbiological culture to confirm the absence of infection.

Reagents

All cell incubations were performed in alpha-minimal essential medium (αMEM Invitrogen, UK) and glutamine (2 mM), benzyl penicillin (100 IU/ml) streptomycin (10 µg/ml) and 10% heat-inactivated fetal bovine serum (FBS). SF cell cultures were incubated in a humidified atmosphere with 5% CO₂ at 37°C. Human RANKL was purchased from Peprotech Europe, UK. Human OPG, M-CSF, TNFα, and IL-1α were purchased from R&D Systems Europe, (Abingdon, UK).

Isolation and culture of SF macrophages

SF was aspirated and collected in sterile 50 ml universal tubes, then centrifuged at 2250 r.p.m. for 25 min at 4°C. The cell pellet was removed, washed in αMEM and resuspended in αMEM/FBS. The number of cells in the cell suspension was counted in a haemocytometer after lysis of red cells with a 5% v/v acetic acid solution. SF cells (1 x 10⁶) were added to 4 mm diameter dentine slices and 6 mm diameter glass coverslips in 96-well plates and incubated for 2 h at 37°C in 5% CO₂. After 2 h incubation, non-adherent cells were then removed by washing in αMEM. Dentine slices and glass coverslips were then transferred to 16 mm diameter wells of a 24-well tissue culture plate and incubated for up to 14 days in αMEM supplemented with 10% FBS in the presence and absence of human M-CSF (25 ng/ml) and soluble RANKL (30 ng/ml) or human M-CSF (25 ng/ml) with TNFα (20 ng/ml) and IL-1α (10 ng/ml) (±OPG, 100 ng/ml). The culture medium and respective supplements were replaced every 3 days.

Cytochemical assessment of osteoclast formation

Cell preparations on coverslips at 24 h, 72 h, 7 days and 14 days were assessed histochemically for the expression of the osteoclast-associated enzyme, tartrate-resistant acid phosphatase (TRAP). Histochemical staining for TRAP was carried out using a commercially available kit (Sigma-Aldrich, UK). Cell preparations were fixed in citrate/acetone solution and stained for acid phosphatase, using naphthol AS-BI phosphate as a substrate in the presence of 1.0 m tartrate; the product was reacted with fast garnet GBC salt [15].

Cell preparations on coverslips were also stained immunohistochemically by an indirect immunoperoxidase technique with the monoclonal antibodies 23C6 (Seropec, UK), which is directed against CD51, the vitronectin receptor (VNR), a highly osteoclast-associated antigen [16], 3G5 a mouse anti-human CD44 (anti-CD44 v3, subclass IgG2a) (R&D Systems Europe, UK) and Tuk 4 (Dako, UK), which is directed against CD14, a monocyte antigen which is not expressed by osteoclasts [17]. The cell preparations on coverslips were counterstained with haematoxylin.

Functional evidence of osteoclast differentiation

Lacunar resorption

Functional evidence of osteoclast differentiation was obtained by a lacunar resorption assay system in which cells are cultured on dentine slices as described previously [3]. Dentine provides a smooth-surface mineralized substrate for the identification of lacunar resorption pits, which are only formed by osteoclasts.

After SF cells were cultured on dentine slices for 24 h, 7 days and 14 days, the slices were removed from the wells and fixed in 4% glutaraldehyde and 10% Triton X-100. The dentine slices were dehydrated by passing through graded alcohols and then through graded hexamethyl–disilazane solution (Sigma-Aldrich, UK), as previously described [18], before being air-dried. Dentine slices were then mounted onto aluminium stubs (Agar Scientific Ltd, UK) using double-sided sellotape, sputter-coated with gold and examined using a Philips SEM 505 scanning electron microscope. The extent of lacunar resorption on the dentine slice was determined by point counting, as previously described [19,20]. The results are expressed as the percentage of surface area resorbed ± standard deviation of the mean. The number of points lying over resorption pits were measured. All measurements were
carried out at ×1000 magnification to ensure accurate results; at least three dentine slices were examined for each treatment.

F-actin

When osteoclasts are cultured on a glass or dentine surface, multiple rows of podosomes are localized in the area corresponding to the sealing zone of osteoclasts [21,22]. Podosomes consist of an F-actin core surrounded by the actin-binding proteins vinculin, talin, gelsolin and α-actinin [23]. To detect F-actin ring structure [24], SF macrophages on coverslips and dentine slices were fixed with 4% formaldehyde for 5 min and permeabilized for 6 min in 0.5% Triton X-100 (in PBS) and rinsed with PBS. The cells on dentine slices were then incubated with TRITC-conjugated phalloidin (Sigma-Aldrich, UK) for 20 min and observed using a conventional fluorescence microscope.

Statistical analysis

Each experiment was carried out in triplicate. Statistical significance was determined using Student’s t-test and p values <0.05 were considered significant.

Results

Phenotypic characteristics of isolated SF macrophages

CD14+ mononuclear cells were noted in all centrifuged specimens of SF from OA, RA and PPA joints examined. Numerous neutrophil polymorphs were present in the 24 h cultures of RA and PPA SF cells but were not present in 72 h cultures on glass coverslips. More than 95% of cells in 72 h cultures expressed the monocyte/macrophage marker CD14 (Figure 1a). These cells also expressed CD44 (Figure 1b) but were negative for the osteoclast markers TRAP and VNR (Figure 2a). 24 and 72 h cultures of SF cells on dentine slices showed no evidence of lacunar resorption (Figure 2b), neither did 14 day cultures of SF cells without added factors. Isolated SF cells that were maintained in culture after 24 h thus did not express the cytochemical or functional phenotype of osteoclasts.

RANKL-induced osteoclast formation from SF macrophages isolated from OA, RA and PPA joints

In 14 day cultures on glass coverslips of SF macrophages derived from OA and RA joints, TRAP+ and
VNR⁺ multinucleated cells were formed when SF cells were incubated with 10% FBS in the presence of M-CSF and RANKL (Figure 3a, b). F-actin ring formation (Figure 3c) was also noted when these cells were incubated for 14 days on dentine slices and glass coverslips. TRAP⁺ and VNR⁺ cells formed in OA SF macrophage cultures were small (10–20 µm) and had fewer than four nuclei. RA SF macrophage cultures also contained numerous TRAP⁺ and VNR⁺ cells but 30% of the cells were large and multinucleated (30–70 µm), with some cells containing up to 30 nuclei (Figure 3a, b); fewer multinucleated TRAP⁺ and VNR⁺ cells were noted in OA cultures than in RA cultures. The size of the SF cells cultured from OA and RA joints was confirmed by scanning electron microscopy (SEM). Cells cultured from RA joints were large, rounded or flattened and spread over the dentine surface, to which they were attached by fine microvilli (Figure 4a). Some cells had cytoplasmic processes that extended up to 20 µm over the dentine surface. RA SF macrophage cultures also contained numerous small cells (<30 µm) of similar morphology to those described above. These small cells were also very numerous in OA SF macrophage cultures.

In cultures of SF macrophages from PPA joints, incubated for 14 days with RANKL and M-CSF, large numbers of TRAP⁺/VNR⁺, F-actin ring-forming cells were also noted (Figure 3d–f). These TRAP⁺/VNR⁺ cells were almost all small (10–20 µm) and mononuclear or binucleated. By SEM examination it was evident that cells in the 14 day cultures were small (<20 µm maximum dimension) and had numerous surface ruffles; these cells were commonly aggregated together and were associated with resorption pit formation (Figure 4e, f).

Lacunar resorption was not seen in cultures of SF macrophages incubated for 1, 3 or 7 days. Lacunar resorption was first noted after 14 days of incubation in RANKL-treated cultures; in the absence of treated cultures no lacunar resorption was seen in 14 day cultures in all OA, RA (Figure 4b) and PPA SF macrophage cultures (Figure 4d–f). The mean percentage of lacunar resorption was significantly increased in PPA and RA SF macrophage cultures compared with OA SF macrophage cultures (p < 0.005, p < 0.001 respectively) (Figure 5). In RA and OA SF macrophage cultures, resorption lacunae were frequently large and compound, being composed of more than one area of lacunar excavation (Figure 4b). Resorption pits formed in PPA SF macrophage cultures were generally small, discrete, round or ovoid areas of lacunar excavation, some of which did not possess a well-defined margin (Figure 4d). These pits were approximately of the same dimensions as the small TRAP⁺/VNR⁺ cells noted in PPA cultures on glass coverslips. Much of the dentine surface was covered with these small pits and in some areas it appeared that numerous small areas of resorption had coalesced to form large areas of lacunar excavation (Figure 4e, f).

Figure 3. SF macrophages extracted from RA joints after 14 days in culture with MCSF (25 ng/ml) and RANKL (30 ng/ml) resulted in the formation of large multinucleated TRAP⁺ (a), VNR⁺ (b) cells which formed F-actin rings (c). SF macrophages extracted from PPA joints after 14 days in culture with RANKL (30 ng/ml) and M-CSF (25 ng/ml) resulted in the formation of numerous small (10 µm mononuclear) TRAP⁺ (d) and VNR⁺ (e) cells, which formed F-actin rings (f). Bars represent 15 µm (a, b, c) or 5 µm (d, e, f).
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Figure 4. Scanning electron micrographs of dentine slices after culturing for 14 days with SF macrophages from RA (a, b) and PPA (d–f) joints treated with MCSF (25 ng/ml) and RANKL (30 ng/ml) or from RA (c) treated with MCSF (25 ng/ml) and TNFα (20 ng/ml) IL-1α (10 ng/ml). Bars represent 10 µm (a, c, d, f) or 50 µm (b, e). (a) Macrophage showing attachment to the dentine with formation of broad pseudopodia and cytoplasmic projections (arrows). (b) Example of an extensive resorption pit formed by SF macrophages from RA cultured with RANKL. (c) Example of the small individual pits formed by SF macrophages from RA cultured with TNFα. (d) A small SF macrophage from a PPA joint and treated with RANKL showing associated shallow lacunar excavations with no distinct margins. (e) Low-power image showing the extensive lacunar formation by SF macrophages from PPA joints treated with RANKL. (f) Enlargement showing many small macrophages associated with the large lacunae.

TNFα-induced osteoclast formation from SF macrophages isolated from RA and PPA joints

Cultures of SF macrophages from RA joints, incubated for 14 days in the presence of M-CSF and TNFα, also contained TRAP+ and VNR+ cells capable of F-actin ring formation. The number of TRAP+ and VNR+ cells were fewer than in RANKL-treated cultures and generally contained one to three nuclei. No TRAP+ and VNR+ cells were observed in OA SF macrophage cultures derived from OA joints incubated for 14 days in the presence of M-CSF and TNFα. Cultures of SF macrophages derived from PPA joints also contained TRAP+ and VNR+ cells, but as in RANKL-treated cultures most of these cells were small and mononuclear.

In cell cultures incubated with TNFα/IL-1α for 14 days on dentine slices, lacunar resorption pits were noted in SF macrophage cultures derived from RA and PPA but not OA joints. TNFα/IL-1α-induced lacunar resorption in RA and PPA SF macrophage cultures was characterized by the formation of small discrete resorption pits, which measured up to 10 µm in diameter (Figure 4c). The area of lacunar resorption in RA and PPA SF macrophage cultures was much less in TNFα-treated cultures than in RANKL-treated cultures. Moreover the mean percentage of lacunar resorption was significantly increased in PPA and
Figure 5. Mean percentage lacunar resorption in OA, RA and PPA-derived SF macrophages after treatment with M-CSF (25 ng/ml) and soluble RANKL (30 ng/ml) or M-CSF (25 ng/ml) with TNFα (20 ng/ml) and IL-1α (10 ng/ml) (±OPG, 100 ng/ml). Error bars represent the standard deviation of the mean. The statistical differences between treated groups is given on the figure (*p < 0.005, **p < 0.001 and ***p < 0.005 relative to corresponding RANKL or TNFα-treated OA SF macrophage cultures)

RA SF macrophage cultures compared with OA SF macrophage cultures (p < 0.001 and p < 0.005, respectively) (Figure 5). TNFα/IL-1α-treated cultures did not contain the large compound resorption pits or confluent areas of lacunar excavation that were noted in SF macrophage cultures incubated with RANKL and M-CSF.

Discussion

In this study, we have shown that SF macrophages found in OA, RA and PPA joints are capable of differentiating into osteoclasts that are capable of lacunar resorption. RA and PPA macrophages differentiated into osteoclasts via both RANKL and TNFα signalling pathways, whereas OA macrophages differentiated into osteoclasts only through the RANKL pathway. Osteoclasts formed from SF macrophages were generally smaller than normal osteoclasts but were capable of extensive lacunar resorption. Increased osteoclast formation and resorption was seen in RA and PPA SF macrophage cultures, as compared to OASF macrophage cultures, suggesting that one means whereby marginal and subchondral erosions could occur in these conditions is by SF macrophage–osteoclast differentiation.

We found that SF macrophages that express the monocyte/macrophage antigen, CD14, were capable of differentiating into osteoclasts. Although adherent SF cells are known to consist of two subpopulations, round-shaped macrophage-like cells (CD68+) and spindle-shaped fibroblast-like cells (Thy-1+), in our experiments almost all of the cultured cells were CD14+ macrophage-like cells (Figure 2a, b). Macrophage-osteoclast differentiation was determined by the acquisition of a number of osteoclast markers including TRAP+, VNR+, F-actin ring formation and the ability to carry out lacunar resorption. We established that SF macrophages from OA, RA and PPA joints were capable of differentiation into osteoclasts in the presence of both M-CSF and RANKL. Takano et al., using a very different culture system, also found that CD14+ SF cells from OA and RA joints were capable of osteoclast differentiation when stimulated with RANKL [26]. Employing long-term cultures of adherent and non-adherent cells isolated from OA and RA joints, they found that RANKL-induced resorption was stimulated by a number of cytokines and growth factors including IL-3, IL-5, IL-7 and GM-CSF [26]. They also showed that osteoclast formation and resorption could be stimulated by culturing SF cells in these growth factors. However, it was not certain whether these factors alone stimulated osteoclastogenesis or...
whether this occurred through the RANKL pathway, as these cell cultures contained adherent and non-adherent cells, including synovial fibroblasts, which express RANKL, and it was not established whether OPG could abolish osteoclast formation and resorption under these conditions. They also observed more resorption in SF cell cultures derived from RA compared with OA joints; these data and the findings of our present study would strongly support the concept that SF macrophages are capable of osteoclast differentiation by a RANKL-dependent process.

In this study, we also observed that SF macrophages isolated from RA and PPA (but not OA) joints could differentiate into osteoclasts via a RANKL-independent mechanism using TNFα/IL-1α. TNFα has been shown to induce osteoclastogenesis in RANK knock-out mice (RANK−/−) [27] and to promote the formation of multinucleated cells from rodent osteoclast precursors present in the bone marrow macrophage population [28]. We have previously shown that TNFα and IL-1α can induce osteoclastogenesis from mononuclear precursors present in the CD14+ monocyte fraction of human peripheral blood [13]. This RANKL-independent mechanism of osteoclast formation was not inhibited by the addition of OPG was but abolished by the addition of an antibody to the TNFα receptor [13]. TNFα has been shown to increase RANKL expression by osteoblasts and bone stromal cells, but these cells are not present in the SF macrophage cultures we employed. In any case, the possibility that these cells could have been present in the TNFα/IL-1α-treated SF macrophage cultures, and were capable of producing sufficient RANKL to induce macrophage–osteoclast differentiation, was excluded by the fact that adding OPG to these cultures did not abolish osteoclast formation and resorption. Few compound resorption pits or large areas of lacunar excavation were produced in TNFα/IL-1α-treated SF macrophages; this pattern of lacunar resorption, which is unlike that seen in RANKL-treated cultures, has previously been described in human monocyte cultures to which these cytokines have been added [13].

The TNFα receptors soluble TNF-R55 and TNF-R75 are known to be up-regulated in RA SF compared with OA SF [29], which results in a decrease of TNFα biological activity [30]. A similar up-regulation of the receptors on SF cells will have opposite effects; increase TNFα biological activity in RA as opposed to OA SF macrophages. This may partly explain the ability of RA as opposed to OA SF macrophages being more responsive to TNFα/IL-1α stimulation. Differences between synovial macrophage populations in OA and inflammatory arthritis have been documented in terms of expression of macrophage activation markers, with increased production of M-CSF [31], IL-17 [32], RANKL [11], IL-13 [33], TNFα [34] and HGF [35] by RA synovial macrophages being reported. It is possible that this increase in activation of RA compared with OA macrophages is reflected in differences in the ability of SF macrophage osteoclast precursors to differentiate into functional osteoclasts.

This is the first study to document increased osteoclast formation and lacunar resorption by SF macrophages isolated from joints affected by crystal arthritis. Large numbers of small, mainly mononuclear TRAP+/VNR+ cells, which were capable of extensive resorption, were formed in cultures of SF macrophages derived from PPA joints. Crystal-related joint diseases have been associated with increased TNFα, IL-6, IL-8, TGFβ and prostaglandin production [36–39]. These cytokines and growth factors are known to promote osteoclast formation and resorption activity. Calcium pyrophosphate dihydrate (CPPD) crystals have been shown to promote the survival of murine bone marrow-derived macrophages and the synthesis of DNA in the presence of low (suboptimal) concentrations of M-CSF [40,41], suggesting that one means whereby CPPD may contribute to increased osteoclast formation is through increased survival of mononuclear phagocyte osteoclast precursors [41].

Mononuclear cells that exhibit the cytochemical and functional characteristics of osteoclasts, including the ability to carry out lacunar resorption, have previously been noted in mouse and human macrophage cultures [42–44]. It is notable, however, that in long-term cultures of SF macrophages isolated from all the joints we examined, many of the cells, including those associated with lacunar resorption, were small and mononuclear or binucleated. Most strikingly there were very large numbers of actively resorbing mononuclear cells in SF cultures of macrophages isolated from PPA joints. It is not clear why the resorbing cells which differentiated from SF macrophages remained mononuclear in the cultures treated with MCSF and RANKL, the growth factors required for osteoclast differentiation, but one major difference between SF macrophages and blood monocytes/tissue macrophages is that they are harvested from a fluid microenvironment that contains abundant hyaluronic acid (HA). HA binds to CD44 and occupancy of the CD44 receptor by HA is known to prevent multinucleation of macrophages [42]. Antibodies to CD44 are known to inhibit osteoclast formation without altering osteoclast resorption activity [45]. It is thus possible that the CD14+/CD44+ SF macrophages, which we isolated from PPA and other joints, remained largely mononuclear in culture as the CD44 receptor, which is indispensable for cell fusion [46,47], is occupied by large amounts of its ligand HA or HA fragments [48]. It is possible that CD44 and soluble CD44 up-regulation in RA [49] comparing to OA may allow for RA macrophages to fuse and become large multinucleated cells. Differences in HA and other specific SF constituents are known to exist in inflammatory and non-inflammatory arthritic conditions, but whether these affect osteoclast formation merits further investigation.
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References

37. Liu R, O’Connell M, Johnson K, Pritzker K, Mackman N, Terkelbaur R. Extracellular signal-regulated kinase 1/2 extracellular...


