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ORIGINAL ARTICLE

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Stimulation of osteoclast formation by inflammatory synovial fluid

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Abstract Peri-articular bone resorption is a feature of arthritis due to crystal deposition and rheumatoid disease. Under these conditions, the synovial fluid contains numerous inflammatory cells that produce cytokines and growth factors which promote osteoclast formation. The aim of this study was to determine whether inflammatory synovial fluid stimulates the formation of osteoclasts. Synovial fluid from rheumatoid arthritis (RA), pyrophosphate arthropathy (PPA) and osteoarthritis (OA) patients was added to cultures (n=8) of human peripheral blood mononuclear cells (PBMCs) in the presence and absence of macrophage colony-stimulating factor (M-CSF) and the receptor activator of NF-κB ligand (RANKL). Osteoclast formation was assessed by the formation of cells positive for tartrate-resistant acid phosphatase (TRAP) and vitronectin receptor (VNR) and the extent of lacunar resorption. The addition of 10% OA, RA and PPA synovial fluid to PBMC cultures resulted in the formation of numerous multinucleated or mononuclear $TRAP^+$ and VNR^+ cells which were capable of lacunar resorption. In contrast to PBMC cultures incubated with OA synovial fluid, there was marked stimulation of osteoclast formation and resorption in cultures containing inflammatory RA and PPA synovial fluid which contained high levels of tumour necrosis factor alpha, a factor which is known to stimulate RANKL-induced osteoclast formation.

Keywords Synovial fluid · Rheumatoid arthritis · Crystal · Pyrophosphate · Osteoclast

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Introduction

Bone resorption is a common feature of a number of inflammatory joint conditions including rheumatoid arthritis (RA) and crystal (e.g. urate, pyrophosphate) arthropathy. In both RA and acute crystal arthritis, the synovial fluid is characterised as inflammatory; it contains abundant neutrophil polymorphs and other leucocytes, including macrophages and lymphocytes [12, 29]. These activated inflammatory cells produce abundant cytokines and growth factors as well as proteolytic enzymes and immune components that are known to promote osteoclastic bone resorption [11].

Osteoclasts are multinucleated cells which are specialised to carry out lacunar bone resorption [4]. Osteoclasts are formed by the fusion of mononuclear osteoclast precursors that are found in the bone marrow and the monocyte fraction of peripheral blood; osteoclasts can also be formed from tissue macrophages, including synovial macrophages [13, 14]. Osteoclast formation occurs in the presence of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κ beta ligand (RANKL), a tumour necrosis factor (TNF)-related protein [26, 33]. RANKL is expressed on the membrane of osteoblasts, some fibroblasts (e.g. synovial fibroblasts) and inflammatory cell (e.g. T lymphocyte) populations, and is released locally as a soluble factor [16]. Osteoclastogenesis involves binding of RANKL to the receptor activator of NF-κB (RANK), which is present on osteoclast precursors. This process is inhibited by osteoprotegerin (OPG), a

soluble decoy receptor for RANK, which is produced by many cells, including osteoblasts, synovial fibroblasts and T lymphocytes [16, 37, 40].

The concentration of inflammatory cytokines, such as TNFα and interleukin (IL)-1, is elevated in the synovial fluid of various forms of inflammatory arthritis [28, 32]. This is of interest because it has recently been shown that inflammatory cytokines can influence the expression of RANKL and OPG by bone stromal cells [15, 16]; this cytokine effect could account for the recent observation that the soluble RANKL to OPG ratio is increased in the synovial fluid of patients with RA compared with those with OA [22]. The increase in inflammatory cytokines found in various forms of inflammatory and non-inflammatory arthritis may also directly contribute to osteoclast formation and bone resorption under these conditions because it has recently been shown that cytokine factors, such as TNFα, IL-6 and IL-11, are capable of inducing osteoclast differentiation (in the presence of M-CSF) by a RANKL-independent mechanism [8, 21, 24, 25].

In this study, we have sought to determine the effect of inflammatory and non-inflammatory synovial fluid on osteoclast formation and lacunar resorption. We have analysed the effect of synovial fluid taken from uninflamed OA joints and compared it with that of inflammatory synovial fluid taken from joints affected by RA and crystal (pyrophosphate) arthritis on monocyte–osteoclast differentiation. Our findings indicate that inflammatory synovial fluid is capable of stimulating osteoclast formation and that, in this way, it may contribute to the joint destruction that occurs under these conditions.

Materials and methods

Reagents

All cell incubations were performed in alpha minimal essential medium (αMEM) (Gibco, UK) supplemented with glutamine (2 mM), benzyl penicillin (100 IU/ml) and streptomycin (10 μg/ml). Positive control cultures were supplemented with 10% foetal bovine serum (FBS). Test cultures were supplemented with 10% RA, pyrophosphate arthritis (PPA) or OA synovial fluid. Cell cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2. Hyaluronidase was purchased from Sigma-Aldrich (UK). Amgen Inc. (Thousand Oaks, CA, USA) kindly provided the soluble RANKL. Human M-CSF was purchased from R&D Systems (UK).

Preparation of synovial fluid

Synovial fluid was aspirated from the hip and knee joints of five seropositive RA patients (five women; average age, 49.3 years), six OA patients (four women and two men; average age, 67.7 years) and three PPA patients (one woman and two men; average age, 66.6 years). The patient diagnoses of OA, RA and PPA were made on the basis of clinical, radiological and relevant serological examination as well as histology of the synovial tissues (Fig. 1) [2]. Using the grading system of Krenn et al. [23] for the degree of synovitis, the inflammatory changes in the joints examined ranged from 0 to 1 in OA, 2 to 3 in RA and 1 to 3 in PPA. Each synovial fluid was defined as inflammatory or non-inflammatory on the basis of the white blood cell count per cubic millimetre (WBC/mm^3) as previously described [12, 29]; non-inflammatory synovial fluids contained less than 1,500 WBC/mm^3, and inflammatory synovial fluids contained more than 1,500 WBC/mm^3. The presence of typical, positively birefringent crystals in PPA synovial fluid samples was confirmed by polarization microscopy. A sample of each fluid was also sent for microbiological culture to confirm the absence of infection. Each synovial fluid sample was incubated with hyaluronidase (1,000 U/ml) at 37°C for 45 min and then centrifuged at 700×*g* for 10 min to remove all cells.

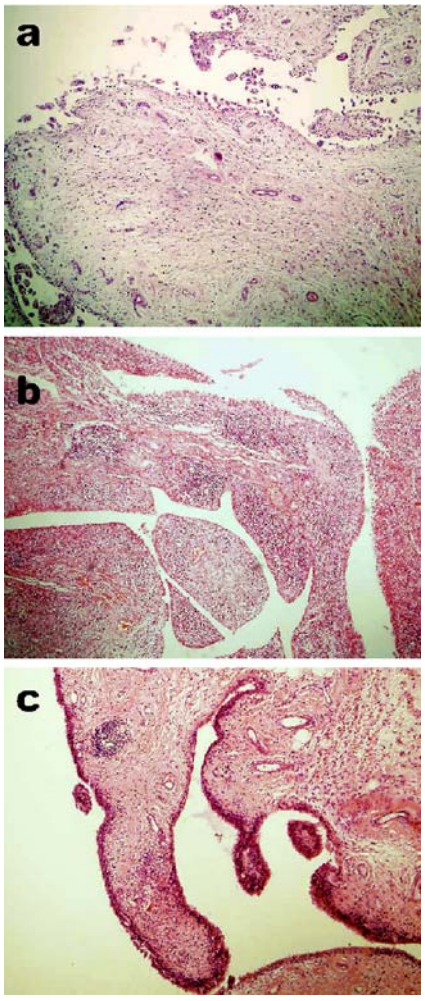

Fig. 1 Representative histology of **a** OA, **b** RA and **c** PPA synovium of joints from which synovial fluid was obtained. In RA and PPA synovium, there is, respectively, a diffuse and focal chronic inflammatory cell infiltrate. Haematoxylin and eosin, original magnification ×40

Aliquots of the synovial fluid were then placed in vials and stored at −80°C.

Isolation and culture of human monocytes

The peripheral blood of eight healthy male donors (age 25–40) was collected into tubes containing EDTA, diluted 1:1 in αMEM, layered over Ficoll-Hypaque (Pharmacia, UK) and centrifuged at 693×*g* for 18 min at 4°C. The peripheral blood mononuclear cell (PBMC) layer was removed and washed in αMEM, and the cell pellet was then resuspended in αMEM/FBS. The number of PBMCs in the cell suspension was counted in a haemocytometer after lysis of red cells with a 5% v/v acetic acid solution. PBMCs (2×10^5) were added to 4-mm-diameter dentine slices and 6-mm-diameter glass cover-slips and incubated for 2 h at 37°C in 5% CO_2. After 2 h of incubation, non-adherent cells were then removed by vigorous washing. Dentine slices and glass cover-slips were then transferred to 16-mm-diameter wells of a 24-well tissue culture plate and incubated for up to 21 days in αMEM supplemented with 10% RA, PPA or OA synovial fluid in the presence and absence of human M-CSF (25 ng/ml) and RANKL (30 ng/ml). Positive controls consisted of PBMCs cultured in the presence of 10% FBS with M-CSF (25 ng/ml) and RANKL (30 ng/ml). The culture medium and respective supplements were entirely replaced every 3 days.

Analysis of CD44 receptor expression on human PBMCs by flow cytometry

Cells were washed once in 1 ml wash buffer (PBS containing 0.5% BSA), resuspended in 100 μl wash buffer and labelled with mouse anti-human CD44 (R&D Systems) or an isotype control mouse IgG2 for 30 min. After washing, cells were labelled with a rabbit anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Dako) for 30 min. Cells were then washed twice in PBS and resuspended in 300 μl PBS for flow cytometric analysis. The level of FITC fluorescence was measured using a FACSCalibur flow cytometer (Becton Dickinson).

Cytochemical assessment of osteoclast formation

Cell preparations on cover-slips at 24 h 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, 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 [30]. Cell preparations on cover-slips were also stained immunohistochemically by an indirect immunoperoxidase technique with the monoclonal antibody 23C6 (Serotec, UK), which is directed against CD51; the vitronectin receptor (VNR), a highly osteoclast-associated antigen [10]; 3G5, a mouse anti-human CD44 (anti-CD44 v3, subclass IgG_{2a}) (R&D Systems Europe) expressed by PBMC and osteoclasts; Tuk 4 (Dako, Japan), which is directed against CD14, a monocyte antigen which is not expressed by osteoclasts [5, 20]; and M7254 (Dako, UK), which is directed against CD3, a T-cell marker. The cell preparations on cover-slips were counterstained with haematoxylin. Positive controls for $CD14^+$ and $CD44^+$ staining consisted of cell preparations on cover-slips of peripheral blood monocytes and, for VNR staining, osteoclasts from giant cell tumour of the bone. Negative controls consisted of cover-slips with no added primary antibody and isotype control for each antibody (IgG_2 or IgG_1).

Functional evidence of osteoclast differentiation

Functional evidence of osteoclast differentiation was determined by a lacunar resorption assay system using cell culture on dentine slices [13, 17]. Dentine provides a smooth-surface mineralised substrate for the assessment of lacunar resorption. After cells had been cultured on dentine slices for 24 h and for 3, 7, 10, 14, 18 and 21 days, the slices were removed from the wells, rinsed in PBS and placed in 0.25% trypsin for 15 min. The slices were then washed vigorously in distilled water and left overnight in 1 M ammonium hydroxide. The dentine slices were then sonicated to remove cell debris and stained with 0.5% toluidine blue. Dentine slices were examined by light microscopy and the percentage surface area of lacunar resorption in each dentine slice then measured using Adobe PhotoShop 5.5 image analysis software.

Ultrastructural evidence of osteoclast differentiation

Ultrastructural evidence of osteoclast differentiation was determined by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). SEM was performed after cells had been cultured on dentine slices for 24 h and for 3, 7, 10, 14, 18 and 21 days. The cell cultures on dentine slices were fixed in a 4% glutaraldehyde solution and then dehydrated by passing through graded alcohols and then through graded hexamethyldisilazane solution (Sigma, UK) before being air dried. The dentine slices were then mounted onto aluminium stubs using double-sided Sellotape, sputtered with gold and examined using a Philips SEM 505 scanning electron microscope. TEM was performed after cells had been cultured on dentine slices for 15 days. The cell cultures were fixed with 2.5% glutaraldehyde/0.1 M cacodylate buffer, pH 7.2, postfixed in osmium tetroxide, dehydrated by passing through graded alcohols and then embedded in epoxy resin. Serial thin sections were cut and then stained with uranyl acetate and lead citrate before examination in a Joel 1200EX transmission electron microscope.

The effect of OA, RA and PPA synovial fluid on osteoclast resorption activity

The effect of OA, RA and PPA synovial fluid on osteoclast resorption activity was determined using cultures of mature osteoclasts obtained from two giant cell tumours of bone. Giant cell tumour tissue was curetted with a scalpel blade and the resultant cell suspension added to dentine slices in 96-well tissue culture plates. Cells were settled on the dentine slices for 2 h, washed in MEM, and then placed in 24-well tissue culture plates containing 1 ml MEM/FCS and 10% RA, PPA or OA synovial fluid. Cell cultures were incubated for 24 h, after which time the extent of lacunar resorption was assessed as described above.

Enzyme-linked immunosorbent assay for OPG/TNFα

Aliquots of synovial fluid were assayed to determine the concentration of OPG and TNFα by an enzyme-linked immunosorbent assay (ELISA) system (Duoset; R&D Systems). The 96-well plates were coated with 2 μg/ml mouse monoclonal anti-human OPG or with mouse monoclonal anti-human TNFα (R&D Systems). The OPG standard curve was generated using recombinant human OPG (R&D Systems) at concentrations from 2,000 to 31.25 pg/ml, and the TNFα standard curve was generated using recombinant human TNFα from 1,000 to 15.65 pg/ml (R&D Systems). The secondary antibodies were biotinylated anti-human OPG and anti-human TNFα (R&D Systems) at 200 ng/ml, and detection was done using streptavidin–horseradish peroxidase (R&D Systems). The reaction was stopped after 30 min of incubation in the dark by addition of 50 μl 2 M H_2SO_4. The plate was read at 450 nm on a Dynatech plate reader using Revelation software.

Statistical analysis

Each experiment was carried out in triplicate. Data are presented as the mean percentage lacunar resorption +/− standard error of the mean. Statistical analysis on measurements of mean percentage area resorbed was performed using Student's *t* test. Values less than $p=0.05$ were considered significant.

Results

Phenotypic characteristics of isolated PBMCs

Isolated PBMCs incubated for 24 h on glass cover-slips, in the presence or absence of synovial fluid, expressed the monocyte/macrophage marker CD14 and were negative for CD3 and the osteoclast markers TRAP and VNR. Twenty-four-hour PBMC cultures on dentine slices showed no evidence of lacunar resorption. Isolated cells thus expressed the cytochemical and functional phenotype of monocytes and not osteoclasts. Flow cytometry and immunohistochemistry showed that isolated PBMCs incubated for 24 and 72 h on glass cover-slips in the presence of M-CSF and RANKL also expressed CD44, an antigen which is associated with substrate adhesion and cell–cell fusion (Fig. 2).

The effect of inflammatory (RA, PPA) and non-inflammatory (OA) synovial fluid on osteoclast formation

In control cultures, PBMCs were incubated for up to 21 days with 10% FBS in the presence of M-CSF and RANKL. Under these conditions, numerous $TRAP^+$ and VNR^+ multinucleated cells were seen in 14-day cultures on cover-slips, and numerous resorption pits were formed in 21-day cultures on dentine slices. Substitution of RA, PPA or OA synovial fluid for either M-CSF or RANKL in these monocyte cultures did not result in the formation of $TRAP^+/VNR^+$ cells.

PBMCs incubated for up to 21 days with 10% RA, PPA or OA synovial fluid, in the presence of M-CSF and RANKL, also showed formation of $TRAP^+$ and VNR^+ cells and evidence of lacunar resorption (Figs. 3, 4 and 5a). In contrast to PBMC cultures incubated in the absence of synovial fluid, where $TRAP^+$ and VNR^+ cells first appeared after 5 and 7 days of incubation, respectively, $TRAP^+$ and VNR^+ mononuclear and multinucleated cells

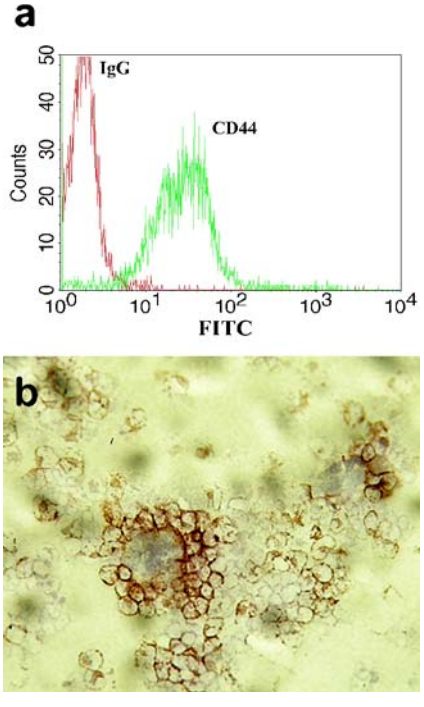

Fig. 2 Seventy-two-hour PBMC cultures incubated with M-CSF and RANKL, analysed for CD44 expression by **a** flow cytometry utilizing antibodies directed against CD44 and its isotype IgG2 and by **b** immunohistochemistry showing expression of the CD44 receptor on mononuclear cells. Original magnification ×400 immunoperoxidase

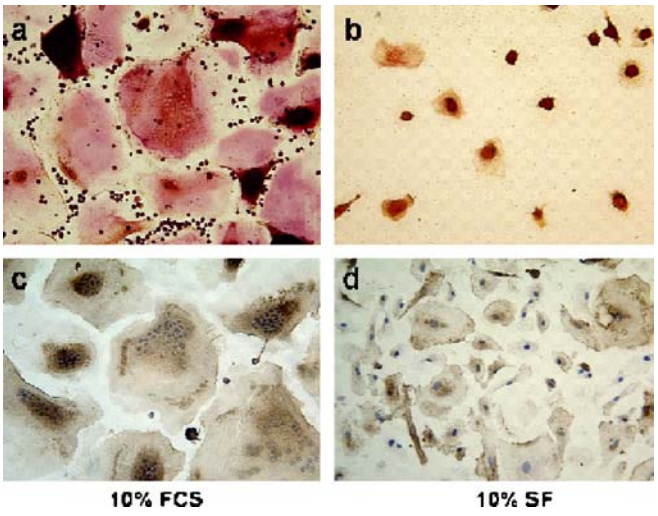

Fig. 3 Fourteen-day PBMC cultures incubated with M-CSF and RANKL, showing **a** large multinucleated $TRAP^+$ cells in control cultures and **b** numerous small largely mononuclear $TRAP^+$ cells, in the presence of 10% RA synovial fluid, and **c** large multinucleated VNR^+ cells in control cultures and **d** small multinucleated mononuclear VNR^+ cells, in the presence of 10% RA synovial fluid. Original magnification ×200

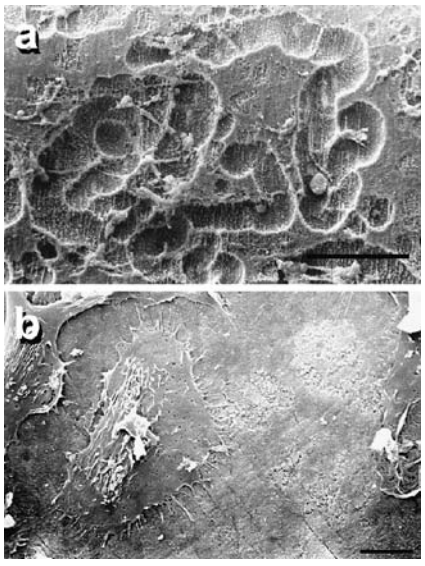

Fig. 5 SEM photomicrograph of an 18-day PBMC culture on a dentine slice incubated with M-CSF, RANKL and 10% RA synovial fluid showing **a** low- and **b** high-power views of lacunar resorption and **c** low-grade surface resorption. Size bar=10 μm

were seen as early as 3 days after PBMC cultures containing 10% OA, RA or PPA synovial fluid were commenced.

More $TRAP^+$ and VNR^+ cells were formed in PBMC cultures to which RA and PPA synovial fluids were added. The majority of $TRAP^+$ and VNR^+ cells present in synovial-fluid-treated cultures appeared mononuclear. Few large $TRAP^+$ and VNR^+ multinucleated cells were formed in PBMC cultures containing synovial fluid, most multinucleated cells being relatively small and containing less than four nuclei. A significant increase in the number of VNR^+ cells was noted in PBMC cultures treated with 10% RA and PPA compared to OA synovial fluid (Fig. 4). The latter was not significantly different from control cultures to which FBS was added. TEM examination of the mononuclear cells formed in PBMC cultures on dentine slices in 10% RA synovial fluid showed that actively resorbing mononuclear cells had a well-developed ruffled border, a characteristic ultrastructural feature of osteoclasts (Fig. 6).

The effect of inflammatory (RA, PPA) and non-inflammatory (OA) synovial fluid on lacunar resorption.

In PBMC control cultures, lacunar resorption on dentine slices was first detected on day 14 of culture (Fig. 5a). However, in PBMC cultures to which OA, RA or PPA synovial fluid was added, resorption began between the seventh and tenth day of incubation. Extensive resorption pit formation was noted in PBMC cultures to which RA and PPA synovial fluids were added; this increased resorption was significant both relative to control PBMC

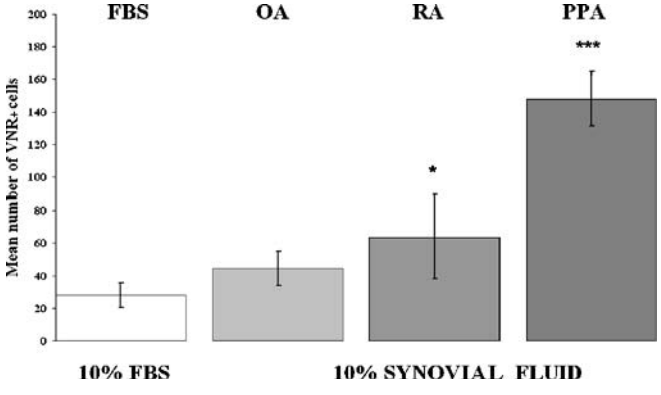

Fig. 4 Mean number of VNR^+ cells formed in 14-day PBMC cultures incubated with M-CSF and RANKL in the presence of 10% FBS (control) and 10% OA, RA and PPA synovial fluid. *p<0.005 and ***p<0.0001 relative to control

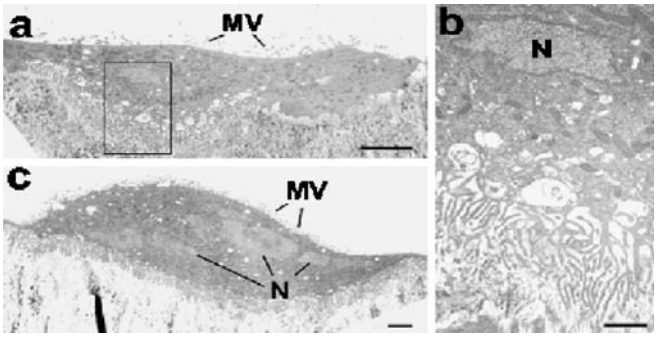

Fig. 6 TEM photomicrograph of an 18-day PBMC culture on a dentine slice incubated with M-CSF, RANKL and 10% RA synovial fluid showing **a** mononuclear cell with a ruffled border [shown in (**b**) at high power] actively resorbing bone and **c** multinucleated cell resorbing bone. *N* nucleus, *MV* microvilli. In (**a**) and (**c**), size bar=10 μm, and in (**b**), the size bar=2 μm

cultures and to cultures containing OA synovial fluid (p=0.03 and p=0.02, respectively) (Fig. 7). Although most resorption seen in the cultures was lacunar in type, a form of non-lacunar resorption characterised by superficial roughening of the dentine surface was also noted both around resorption pits and in direct relation to cells on the bone surface (Fig. 5b). PBMC cultures to which 10% OA synovial fluid had been added showed a similar level of resorption to that seen in control cultures. The addition of synovial fluid from OA, RA and PPA joints had no significant difference on the extent of lacunar resorption by mature osteoclasts isolated from giant cell tumour of bone (Fig. 8); this finding suggests that the increased resorption seen in cultures treated with RA and PPA synovial fluid was due to increased osteoclast formation rather than increased osteoclast resorption activity.

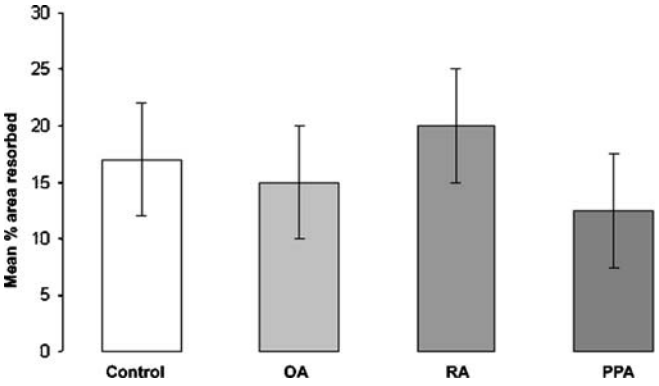

Fig. 8 Mean percentage area resorption produced by mature osteoclasts isolated from giant cell cultures incubated in the presence of 10% non-inflammatory (OA) and inflammatory (RA/PPA) synovial fluid

TNFα and OPG concentration in OA, RA and PPA synovial fluids

Although there was some variation in the concentration of TNFα in the synovial fluid samples taken from individual RA (5.7–20 pg/ml) and PPA (4.7–8.2 pg/ml) joints, the concentration of TNFα in RA and PPA joint synovial fluid was higher than that seen in OA joint fluid, where the TNFα concentration was much less variable (4.3–5.5 pg/ml). Osteoclast formation and lacunar resorption were most pronounced in PBMC cultures to which RA and PPA synovial fluids containing the highest levels of TNFα were added. Only small variations in the concentration of OPG were observed in OA, RA and PPA joints, all fluids tested exhibiting OPG concentrations below 3.5 pg/ml.

Discussion

Mononuclear osteoclast precursors are present in the synovial macrophage population of RA and OA joints, and osteoclast formation by these cells occurs in the presence of M-CSF and RANKL [13, 17]. Variations in the cellular and humoral composition of synovial fluid reflect changes in the synovial tissues with which the fluid is in continuity. Normal synovial fluid is a dialysate of plasma, containing hyaluronan, glycoproteins, albumin and small quantities of larger proteins decreasing in amount with an increase in molecular size. In inflammatory synovial fluid, increasing amounts of all proteins, including high molecular weight proteins found in the plasma, enter the joint. These factors may significantly influence cell differentiation and function, as may cytokines and growth factors produced by activated fibroblasts and leucocytes found in inflamed synovial tissue. RA synovium contains a significant macrophage infiltrate; this is reflected in the high number of macrophages found in the synovial fluid of inflamed RA joints. The extent of synovial macrophage infiltration correlates strongly with the degree of joint erosion in arthritis [42], and increased osteoclast formation has been noted in cultures of synovial macrophages from RA compared with OA joints [17].

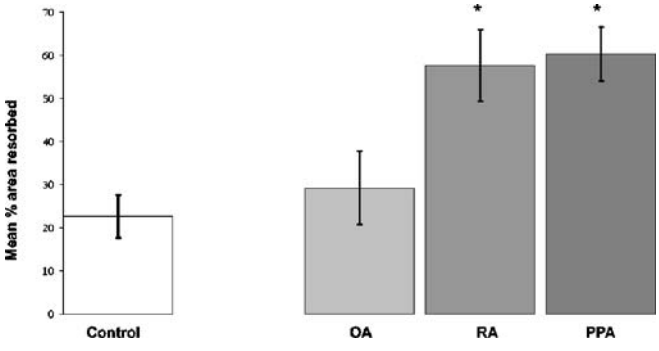

Fig. 7 Mean percentage area resorption produced by osteoclasts formed in PBMC cultures incubated with M-CSF and RANKL in the presence of 10% non-inflammatory (OA) and inflammatory (RA/PPA) synovial fluid. *p<0.05 relative to 10% OA synovial fluid

In the present study, we have shown that the synovial fluid derived from inflamed (RA/crystal arthropathy) joints stimulates RANKL-induced macrophage–osteoclast differentiation. We found that the addition of synovial fluid to monocyte cultures incubated with RANKL and M-CSF resulted in the formation of mononuclear cells and small multinucleated cells that expressed TRAP and VNR, markers of the osteoclast phenotype; these cells possessed a ruffled border and fulfilled the essential functional criterion of osteoclasts, being capable of lacunar resorption. We also found that inflammatory synovial fluid from RA and PPA joints markedly stimulated osteoclast formation and lacunar resorption. In the presence of non-inflammatory OA synovial fluid, osteoclast formation and lacunar resorption were similar to that seen in control cultures, but in the presence of RA and PPA synovial fluid, there was a twofold increase in lacunar resorption relative to OA and control cultures.

In the presence of 10% synovial fluid, many of the $TRAP^{+}/VNR^{+}$ cells in monocyte cultures were either mononuclear or contained fewer than four nuclei. These

cells exhibited cytochemical, functional and ultrastructural characteristics of osteoclasts, being $TRAP^+/VNR^+$ and capable of lacunar resorption pit formation; these cells also had a ruffled border, an area of membrane specialisation which is essential for bone resorption. Mononuclear cells that express the cytochemical and functional characteristics of osteoclasts have previously been noted in cultures of marrow cells and tissue macrophages [6, 7, 34]. Our findings are similar to those of a recent study which found that a significant proportion of bone-resorbing osteoclasts that form in cultures of synovial fluid macrophages are small and contain fewer than four nuclei [1]. The reason for the limited capacity mononuclear phagocytes incubated in synovial fluid to form polykaryons is not known, but it may be related to the fact that synovial fluid contains abundant hyaluronic acid. The CD44 receptor, which we have shown in this study to be expressed by monocytes, is indispensable for cell fusion, and fusion of mononuclear phagocytes is associated with osteoclast differentiation [41]. Since CD44 is the principal receptor for hyaluronic acid [3], it is possible that occupancy of the CD44 receptor by hyaluronic acid and its breakdown products results in a limited capacity of this macrophage population to carry out cell fusion. Occupancy of the CD44 receptor by hyaluronic acid and CD44 antibodies has been reported to inhibit cell fusion without altering osteoclast resorption activity [19, 39].

There was an increase in the number of bone-resorbing cells formed in monocyte cultures to which inflammatory (RA and PPA) synovial fluids were added compared with OA synovial fluid. Although most of the resorption carried out by these cells was lacunar in type, many of the cells exhibited a form of low-grade (non-lacunar) resorption of the dentine surface. This type of resorption has previously been described in arthroplasty-derived and tumour-derived macrophage cultures [18, 35]; however, the extent of the low-grade resorption seen in the monocyte cultures treated with inflammatory synovial fluid was much more extensive than in previous studies, and it is possible that the mononuclear and small multinucleated osteoclastic cells that form in cultures treated with RA and PPA synovial fluid exhibit an increased capacity for this type of resorption.

The resorption activity of mature osteoclasts was not stimulated when inflammatory synovial fluid was added to cultures of osteoclasts derived from giant cell tumours of bone. This suggests that the increased resorption that followed the addition of inflammatory synovial fluid occurred through stimulation of osteoclast formation rather than mature osteoclast activity. Pigmented villonodular synovitis (PVNS) is a condition in which there is periatricular bone resorption associated with heavy macrophage and giant cell infiltrate in synovial tissues. The giant cells in PVNS exhibit the phenotypic characteristics of mature osteoclasts [9, 31]. These osteoclast-like giant cells are most likely formed from synovial macrophages, and stimulation of osteoclast formation under this condition may be similarly promoted by a factor present in PVNS synovial fluid, which is known to contain giant cells.

Levels of RANKL are known to be increased in RA compared with OA synovial fluid [22], but it is unlikely that RANKL alone represents the pro-osteoclastogenic factor in inflammatory synovial fluid because osteoclast formation did not occur when inflammatory synovial fluid was added to monocyte cultures (in the presence of M-CSF). Likewise, although M-CSF has been reported to be increased in RA synovial fluid [38], M-CSF is unlikely to be the factor promoting osteoclast formation in inflammatory synovial fluid because the addition of inflammatory synovial fluid to monocyte cultures (in the presence of RANKL) did not stimulate osteoclast formation and resorption. It has been noted that the level of OPG in synovial fluid is decreased in RA compared with OA joints [22]. We measured the concentration of OPG by ELISA and found in our samples that it was not significantly different in OA, RA and PPA joint fluids. RANKL, however, is known to exist in a soluble form, and it remains possible that the RANKL to OPG ratio could be increased by stimulation of RANKL production by fibroblasts and activated T and B lymphocytes, all of which have been shown to support osteoclast formation in RA [36, 40].

The synovial membrane and synovial fluid in RA and crystal arthritis are known to contain large amounts of TNFα [28, 32], and TNFα has been reported to stimulate RANKL-induced osteoclastogenesis by modulating stromal cell expression of RANKL and OPG [15, 16]. Osteoclast differentiation, however, could not have been stimulated by stromal cells in inflammatory synovial fluid because our cultures contained only monocytes. Recent studies have shown that TNFα can stimulate osteoclast formation from marrow precursors in the presence of permissive levels of RANKL [27], and it is possible that the high levels of TNFα in inflammatory synovial fluid could have stimulated osteoclast formation in this way. Another means whereby TNFα in inflammatory synovial fluid could have stimulated osteoclast formation is through a RANKL-independent mechanism. We and others have shown that TNFα (in the presence of M-CSF) can induce osteoclast formation from mouse marrow precursors and human monocytes and macrophages [8, 21, 24]. We have also recently shown that TNFα can induce osteoclast formation from synovial fluid macrophages isolated from RA and PPA joints [1]. Whether TNFα alone or in concert with other growth factors and cytokines stimulates osteoclast formation by RANKL-dependent and/or RANKL-independent mechanism in inflammatory arthritis is not certain, but a role for TNFα is suggested by our finding that the most extensive resorption was seen in the monocyte cultures to which RA and PPA inflammatory synovial fluids containing the highest levels of TNFα were added.

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