Endogenous MMP-9 and not MMP-2 promotes rheumatoid synovial fibroblast survival, inflammation and cartilage degradation

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Abstract

Objective. The aim of this study was to investigate the effect of endogenous matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9) on the invasive characteristics of RA synovial fibroblasts.

Methods. Synovial fibroblasts isolated from patients with RA or OA were treated with MMP small interfering RNA (siRNA), inhibitors and recombinant proteins or TNF-α, with or without cartilage explants. Cell viability and proliferation were measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide and 5-bromo-2-deoxyuridine (BrdU) proliferation assays, respectively; apoptosis by an in situ cell death detection kit; migration and invasion by CytoSelect invasion assay, scratch migration and collagen gel assays; cartilage degradation by 1,9-dimethylmethylene blue assay; and inflammatory mediators and MMPs by ELISA, western blot and zymography.

Results. MMP-2 was expressed by both OA and RA synovial fibroblasts, whereas only RA synovial fibroblasts expressed MMP-9. Suppressing MMP-2 or MMP-9 reduced RA synovial fibroblast proliferation equally. However, MMP-9 siRNA had greater effects compared with MMP-2 siRNA on promoting apoptosis and suppressing RA synovial fibroblast viability, migration and invasion. Suppression/inhibition of MMP-9 also decreased the production of IL-1β, IL-6, IL-8 and TNF-α, inactivated nuclear factor κB (NF-κB), extracellular signal-regulated kinase (ERK) and c-Jun NH2-terminal kinase (JNK) and suppressed RA synovial fibroblast-mediated cartilage degradation. In contrast, suppression/inhibition of MMP-2 stimulated TNF-α and IL-17 secretion and activated NF-κB, while recombinant MMP-2 (rMMP-2) inactivated NF-κB and suppressed RA synovial fibroblast-mediated cartilage degradation. Results using specific inhibitors and rMMPs provided supportive evidence for the siRNA results.

Conclusion. Endogenous MMP-2 or MMP-9 contribute to RA synovial fibroblast survival, proliferation, migration and invasion, with MMP-9 having more potent effects. Additionally, MMP-9 stimulates RA synovial fibroblast-mediated inflammation and degradation of cartilage, whereas MMP-2 inhibits these parameters. Overall, our data indicate that MMP-9 derived from RA synovial fibroblasts may directly contribute to joint destruction in RA.

Key words: synovial fibroblasts, rheumatoid arthritis, matrix metalloproteinase 2 and 9, proliferation, survival, migration, invasion, cartilage degradation, inflammation.

Introduction

RA is a chronic inflammatory joint disease characterized by hyperplasia of the synovium and cartilage and bone erosion [1]. The normal synovium forms a thin membrane (one to three cell layers) at the edges of joints and provides lubrication and nutrients for the cartilage. In RA, however, this synovial lining layer dramatically increases and transforms into an inflammatory tissue, known as the...
pannus [1]. Pannus expands, attaches to and invades the adjacent cartilage and subchondral bone, causing cartilage degeneration and bone erosion. The major cells in the thickened lining layer and resultant pannus are the activated RA synovial fibroblasts. RA synovial fibroblasts manifest an abnormal phenotype distinguished by increased proliferation, prolonged survival, resistance to apoptosis and invasiveness of adjacent tissues. These changes are often referred to as a tumour-like transformation [1].

Degradation of articular cartilage is one of the early features of RA and is mediated by increased activity of proteolytic systems [2]. In particular, RA synovial fibroblasts exhibit increased production of the matrix-degrading enzyme family [3–5], the matrix metalloproteinases (MMPs). MMPs are a group of zinc-dependent endopeptidases that can degrade every component of the extracellular matrix (ECM) and are the main proteases involved in the invasion and degradation of anatomical barriers [6]. Among the MMPs, the gelatinase subfamily, consisting of MMP-2 (gelatinase A) and MMP-9 (gelatinase B), are especially important in collagen degradation, through digestion of denatured collagen (gelatin), generated by collagenases. Additionally, these two enzymes digest other substrates, including fibrillar collagen I and II [7, 8] and aggrecan, which predominantly exist in cartilage [6].

Although the two gelatinases have similar substrate specificities, there are differences in their mechanism of regulation. MMP-2 is constitutively expressed by most cell types, including synovial fibroblasts [5], and is usually not induced by cytokines or growth factors. In contrast, basal levels of MMP-9 are undetectable in most cell types, except for malignant or inflammatory cells, such as RA synovial fibroblasts [5], and its expression is induced by inflammatory cytokines including TNF-α. In addition to the degradation of ECM, gelatinases can regulate inflammation by processing cytokines/chemokines, with MMP-9 having stimulatory effects and MMP-2 having inhibitory effects on inflammation [9–11].

In RA, MMP-9 is markedly elevated in serum and joint synovial fluid and positively correlates with disease progression and severity [12–14]. MMP-9 knockout mice show reduced severity of antibody-induced arthritis [15]. Although MMP-2 is elevated in the arthritic joint, MMP-2 knockout mice have exacerbated antibody-induced arthritis, indicating that MMP-2 may play a suppressive role in the progression of arthritis [15]. In light of this, a recent study has shown that MMP-2 is able to cleave and inactivate IL-17 [16], an important cytokine regulating the migration/invasion and inflammation of RA synovial fibroblasts [17].

Compared with OA synovial fibroblasts and normal synovial fibroblasts, which produce only MMP-2, one unique feature of RA synovial fibroblasts is the production of both MMP-2 and MMP-9 [5]. To date, the autocrine and paracrine effects of these two MMPs on RA synovial fibroblasts have not been determined. This study defines the ability of endogenous MMP-9 or MMP-2 to regulate proliferation/viability, apoptosis, migration/invasion, inflammation or cartilage degradation in RA synovial fibroblasts. Our results highlight the destructive effect of MMP-9 and the protective effect of MMP-2 produced by these cells.

Methods

Synovial fibroblast isolation and treatment

Human synovial fibroblasts were isolated from synovial tissue obtained from knee replacement surgery of seven patients with RA [5 females, mean age 68.1 years (S.D. 6.2); 2 males, mean age 70.5 years (S.D. 4.7)] and eight patients with OA [3 females, mean age 69.2 years (S.D. 7.2); 5 males, mean age 71 years (S.D. 5.7)] after collagenase digestion, as previously described [5]. The purity of RA synovial fibroblasts was determined by flow cytometry stained with anti-CD68 antibody (eBioscience, San Diego, CA, USA) and anti-fibroblast marker (ER-TR7) (Santa Cruz Biotechnology, Dallas, TX, USA). Cells were used for further experiments if >95% cells were positive for fibroblast marker. Synovial fibroblasts were cultured in DMEM medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and used from passages two to three in all experiments. Before treatment, cells were pre-incubated in DMEM without FBS (basal medium) for 4 h and then transferred to fresh basal medium. Cells were treated with validated small interfering RNA (siRNA) for MMP-2 and MMP-9 as described previously [18], inhibitors for MMP-2 (K_i = 2.4 μM; catalogue no. 444286), MMP-9 (K_i = 2.1 μM; catalogue no. 444293) and a broad-spectrum MMP inhibitor (GM6001, catalogue no. 364206) (Merck KGaA, Darmstadt, Germany); recombinant MMP-2 and MMP-9 (rMMP-2 and rMMP-9) and TNF-α (Sigma, St Louis, MO, USA). Inhibitors were used at optimal concentrations for maximum inhibitory effect without affecting cell viability.

The use of human tissues was approved by the Northern Sydney Local Health District Human Research Ethics Committee. All patients fulfilled the ACR criteria for RA and OA [19, 20] and gave their written informed consent.

siRNA transfection

Synovial fibroblasts were seeded into 6-, 24- or 96-well plates a day before siRNA transfection. Cells were transfected with MMP-2, MMP-9 or scrambled control siRNA using Ribofectin Transfection Reagent (BioCellChallenge, Paris, France) according to the manufacturer’s protocol. The efficacy of siRNA was confirmed at 24 h post-transfection by real-time RT-PCR as described previously [18] and gelatin zymography at 72 h post-transfection.

BrdU cell proliferation assay and MTT assay

Cell proliferation was detected by the 5-bromo-2-deoxyuridine (BrdU) Cell Proliferation assay kit (Merck Millipore, Darmstadt, Germany) according to the manufacturer’s instructions and cell viability was determined by the colorimetric 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay.
Apoptosis detection

Apoptotic cells were detected by an in situ cell death detection kit according to the manufacturer’s instructions (Roche Diagnostics Australia, Castle Hill, NSW, Australia). Apoptotic cells were determined by counting positive staining cells under 20× magnification.

Iavage/migration assays

RA synovial fibroblast invasion was measured using the CytoSelect 24-well cell invasion assay kit (basement membrane, colorimetric format; Cell Biolabs, San Diego, CA, USA) according to the manufacturer’s instructions. Invasive cells on the bottom of the membrane were stained and analysed using light microscopy to count the total cell number in five random fields of the membrane at 20× magnification. Results were expressed as invasive cells per field.

Cell migration was detected using a scratch migration assay, as previously described [21]. To eliminate the effect of cell proliferation, RA synovial fibroblasts were treated with mytomycin C (5 µg/ml) for 4 h. Cell monolayers were then wounded and after 24 h the cells that had migrated to the wound areas were counted under a microscope and expressed as a percentage of controls.

Cell migration/invasion was also examined using a collagen gel assay as described previously [22] with modifications. In brief, RA synovial fibroblasts (2 × 10^5 cells/ml in DMEM) were mixed with an equal volume of type 1 collagen solution (Life Technologies Australia, Victoria, NSW, Australia; 2 mg/ml, neutralized to pH 7.2) at 4°C. The cell-containing solution (20 µl/drop) was rapidly transferred into 24-well plates (4 drops/well) and allowed to solidify for 2 h, after which 0.5 ml of DMEM containing 10% FBS was added to each well and plates were incubated at 37°C. After 48 h, crystal violet was added to the wells and stained cells that had migrated out of the gels were counted under a microscope (at 20× magnification). At least 16 collagen gel drops per experimental group were analysed. Results were expressed as the mean number of cells that had migrated from each collagen gel drop.

1,9-Dimethylmethylen blue assay

RA synovial fibroblasts cultured in a 24-well plate were co-incubated with OA cartilage explants in DMEM for 24 h. Sulphated glycosaminoglycans (SGAGs) released from the cartilage explants were determined by the 1,9-dimethylmethylen blue (DMMB) assay against a standard curve of chondroitin sulphate [23].

ELISA

IL-1β, IL-6, IL-8, IL-17 and TNF-α in cell culture supernatants were measured by ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

Gelatin zymography

MMP-2 and MMP-9 protein secretion and activation in the culture supernatants were measured using gelatin zymography under non-reducing conditions, as described previously [24].

Western blot analysis of signalling molecules

Western blotting was performed using whole cell lysates, as described previously [25]. Rabbit anti-phosphorylated (P) or non-P forms of p38, extracellular signal-regulated kinase (ERK) and c-Jun NH2-terminal kinase (JNK) antibodies (Cell Signalling Technology, Danvers, MA, USA) and mouse anti-nuclear factor xB (NF-xB) p65 subunit antibody (Chemicon International, Temecula, CA, USA) were incubated with membranes at 4°C overnight. After three washes, membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. Immunoreactivity was detected using the enhanced chemiluminescence (ECL) detection system (Amersham, Piscataway, NJ, USA). Anti-human β-actin antibody (Sigma) was used to normalize the equal loading.

Immunohistochemistry

Human synovial tissues were fixed in 10% PBS buffered formalin and immunostaining was performed using mouse anti-human MMP-2 and MMP-9 antibodies (R&D Systems) and the Universal LSAB+ Kit/HRP staining kit (Dako, Glostrup, Denmark). Mouse isotype IgG was used as a negative control. Images were acquired and processed using a Nikon digital camera.

Statistical analysis

Significance was determined using one-way analysis of variance for multiple comparisons followed by Tukey’s test or t-test to compare two means. P-values <0.05 were considered statistically significant.

Results

Both MMP-2 and MMP-9 are required for RA synovial fibroblast growth and survival

Immunostaining of synovial tissues confirmed that there was strong staining of MMP-2 and MMP-9 in the lining layers of RA synovial tissues, while only MMP-2 was positively stained in the lining layers of OA synovial tissues (Fig. 1A). Since major cells in the lining layers of synovial tissues are synovial fibroblasts, we compared MMP expression by cultured RA and OA synovial fibroblasts. Consistent with our previous study [5], RA synovial fibroblasts produced both MMP-2 and MMP-9, while OA synovial fibroblasts secreted only MMP-2 (Fig. 1B). To examine whether these MMPs affect RA synovial fibroblast proliferation/viability, MMP-2 and MMP-9 siRNAs, inhibitors or recombinant proteins were utilized. The specificity and efficacy of siRNAs on RA synovial fibroblasts were confirmed by real-time RT-PCR (see supplementary Fig. S1, available at Rheumatology Online) and gelatin zymography (Fig. 1B). Gene expression of both MMPs, either alone or in combination, was suppressed by ~90% post-transfection for 24 h (see supplementary Fig. S1A, available at Rheumatology Online). The protein levels of MMP-2 or MMP-9 were also dramatically suppressed.
decreased in response to their respective siRNA transfection for 72 h and suppression of either MMP did not affect the protein expression of the other MMP, under control or TNF-α-stimulated conditions (Fig. 1B).

Suppressing MMP-2 or MMP-9 reduced RA synovial fibroblast proliferation by ~20% for each, and suppressing MMP-2 and MMP-9 together showed an additive effect on cell proliferation (Fig. 2A). In OA synovial fibroblasts, suppression of MMP-2 by siRNA resulted in a similar reduction in cell proliferation as shown in RA synovial fibroblasts (Fig. 2B). In RA synovial fibroblasts, MMP-9 and to a lesser extent MMP-2, siRNA transfection significantly reduced cell viability 72 h post-transfection (Fig. 2C). Additionally, the in situ cell death detection assay showed that apoptotic cells were increased by 38%, 89% or 90% in response to MMP-2 siRNA, MMP-9 siRNA or both siRNAs, respectively (Fig. 2D). These data suggest that MMP-9 has a more dominant effect in promoting RA synovial fibroblast survival compared with MMP-2.

To confirm the data, RA synovial fibroblasts were treated with specific MMP-2 or MMP-9 inhibitors. Similar to siRNA results, MMP-2 inhibition reduced cell viability by 18% (Fig. 2E) and the MMP-9 inhibitor by 35%, rMMP-9 (50 ng/ml) stimulated cell viability by 25%, whereas rMMP-2 had no significant effect (Fig. 2E). Inhibition of MMP-2 and MMP-9 simultaneously resulted in a similar reduction in cell viability to MMP-9 inhibition alone. GM6001, a broad-spectrum MMP inhibitor, induced a similar 20% reduction in cell viability (Fig. 2F). In OA synovial fibroblasts, inhibition of MMP-2 resulted in a 20% reduction in viability, while the MMP-9 inhibitor, rMMP-2 and rMMP-9 had no effect on OA synovial fibroblast viability (Fig. 2F). Overall, these data suggest that endogenous MMP-2 contributes to the proliferation and survival of both OA and RA synovial fibroblasts, whereas endogenous MMP-9 plays an important role in maintaining survival of RA synovial fibroblasts but not OA synovial fibroblasts.
RA synovial fibroblasts were transfected with control (Cont), MMP-2 (MMP2) and/or MMP-9 (MMP9) siRNA (10 nM). (A) RA synovial fibroblast invasion after siRNA transfection for 48 h as detected by the CytoSelect 24-well cell invasion assay kit. Cell invasion is expressed as invasive cells/20× field. (B) RA synovial fibroblast migration/invasion after siRNA transfection for 72 h as detected by a collagen gel assay. Cells were stained with haematoxylin and eosin. Cell migration/invasion is expressed as cell numbers migrated out of collagen gels. (C) RA synovial fibroblast migration after siRNA transfection for 72 h and wounded for 24 h as detected by a scratching migration assay. To standardize the position of the wound for photography, small indents were made in the plastic well (indicated by white arrows). Cell migration is expressed as a percentage of control. Data shown on the graph are mean (s.e.m.) (n = 4). Images represent one of four independent experiments. Scale bars: 100 μm.

*P < 0.05 and **P < 0.01 when compared with controls, *P < 0.05. MMP: matrix metalloproteinase; siRNA: small interfering RNA.

MMP-9, and to a lesser extent MMP-2, is associated with RA synovial fibroblast migration/invasion

Increased migration and invasion is a distinct feature of RA synovial fibroblasts [1]. Using a commercial membrane invasion assay, suppression of MMP-2, MMP-9 or both by siRNA resulted in a 25%, 46% and 54% decrease in RA synovial fibroblast invasion, respectively (Fig. 3A). It is noteworthy that MMP-9 siRNA had a significantly greater effect than MMP-2 siRNA. Similar results were obtained using a collagen migration/invasion assay (Fig. 3B). Additionally, a scratch migration assay confirmed that MMP-9, and to a lesser extent MMP-2, contributed to RA synovial fibroblast migration (Fig. 3C). The results from all three assays consistently indicate that MMP-9 is a more potent promoter of RA synovial fibroblast migration/invasion than MMP-2.

MMP-9 promotes inflammatory cytokines and signalling molecules by RA synovial fibroblasts

IL-1β, IL-6, IL-8, IL-17, TNF-α and NF-κB are inflammatory mediators that contribute to the pathogenesis of RA and are associated with the inflammatory and invasive properties of RA synovial fibroblasts [1]. Our results showed that suppression of MMP-2 did not affect the production of IL-6 or IL-8, however, it markedly increased TNF-α by 50%, IL-17 by 60% and NF-κB activation by 150% (Fig. 4C, D and F). Conversely, suppression of MMP-9 reduced the production of IL-6, IL-8 and TNF-α by 20%, 55% and 30%, respectively, and NF-κB activation by 37% (Fig. 4). IL-1β was reduced by ~30% in response to either MMP-2 or MMP-9 siRNA transfection (Fig. 4E). When MMP-2 and MMP-9 were suppressed together, IL-6, IL-8 and IL-1β were inhibited to the same extent as MMP-9 suppression alone (Fig. 4A, B and E), whereas TNF-α and NF-κB were increased by >90% and 40%, respectively (Fig. 4C and F), indicating that inhibition of MMP-2 could abolish the effect of MMP-9 inhibition. To investigate whether the effect of MMP-2 and MMP-9 on cytokine production is via NF-κB, NF-κB inhibitor was used to treat the cells. Results showed that although there was no change in IL-1β, IL-6 and IL-8 production,
IL-17 and TNF-α were reduced by >60% and 50%, respectively, by NF-κB inhibitor (Fig. 4G).

Activation of mitogen-activated protein (MAP) kinases ERK, p38 and JNK is associated with hyperplasia and inflammation of RA synovial fibroblasts [26]. MMP-9 siRNA suppressed the activation of ERK and JNK by 40% and 35%, respectively, whereas MMP-2 siRNA did not significantly affect MAP kinase activation in RA synovial fibroblasts (Fig. 5A). In agreement, rMMP-9 stimulated the activation of JNK and ERK, whereas rMMP-2 had no effect. Activation of NF-κB was suppressed by rMMP-2 but enhanced by rMMP-9 (Fig. 5B). Simultaneous stimulation with rMMP-2 and rMMP-9 showed a similar trend to stimulation by rMMP-9 alone (Fig. 5B). Expression and activation of p38 was not affected by MMP-2 or MMP-9.

MMP-9 and MMP-2 in RA synovial fibroblast-mediated cartilage degradation

Cartilage degradation was measured by SGAG release from human OA cartilage explants. Suppression of MMP-9 by its siRNA or specific inhibitor decreased SGAG release by ~20%, whereas inhibition of MMP-2 showed no effect (Fig. 6A and B). In contrast, rMMP-2 inhibited SGAG release by ~20% and rMMP-9 had no effect in the presence or absence of their respective siRNAs (Fig. 6A and B). Unexpectedly, simultaneous suppression of MMP-2 and MMP-9 caused a 20% increase in SGAG, and this effect could not be reversed by rMMP-2 and rMMP-9 (Fig. 6A).

To investigate the mechanisms associated with RA synovial fibroblast-mediated cartilage degradation, cells were pretreated with specific inhibitors for NF-κB, ERK, p38 and JNK before adding cartilage explants. Inhibition of NF-κB, p38 and JNK, but not ERK, significantly reduced SGAG release by 27%, 23% and 36%, respectively (Fig. 6C).

**Discussion**

We have previously shown that RA synovial fibroblasts, but not OA synovial fibroblasts or normal synovial fibroblasts, produce high levels of MMP-9 at early passage that is depleted after passage 4 [5]. The current study reveals that this endogenous MMP-9 contributes to the prolonged survival and invasive and inflammatory properties of RA synovial fibroblasts.

RA synovial fibroblasts are a dominant cell type in RA synovium and mediate the persistent inflammation as well as cartilage and bone destruction [27]. These cells maintain their aggressive phenotype towards cartilage in vitro and can degrade co-implanted human cartilage in vivo in the absence of inflammation [28]. The in vitro invasiveness of RA synovial fibroblasts is correlated with clinical joint destruction from individual patients with RA [29]. However, the phenotype of RA synovial fibroblasts changes substantially after four passages in culture [30], which occurs simultaneously with the disappearance of MMP-9, providing evidence of an association between MMP-9 and the aggressive behaviour of RA synovial fibroblasts.

Within RA synovium, MMP-9 is localized to sites of inflammation comprising surface synovial lining cells, endothelium and leucocytes [12], and is directly related to the degree of inflammation [12, 13]. MMP-9 gene knockout mice are significantly less susceptible to arthritis [15, 31], resistant to experimental autoimmune encephalomyelitis [32] and have an attenuated inflammatory response to colitic agents [33]. In collagen-induced arthritis, elevated pro-inflammatory mediators such as TNF-α promote MMP-9 activity in local inflammatory cells, such as RA synovial fibroblasts, leading to bone damage [34]. Our immunostaining confirmed that MMP-9 is strongly expressed by synovial lining layers within RA but not OA synovium. In our culture conditions, MMP-9 promoted inflammation in RA synovial fibroblasts.
The current study shows that endogenous MMP-9 can stimulate the secretion of TNF-α, IL-6 and IL-8 (Fig. 4). TNF-α and IL-6 are key determinants for inflammation and bone erosion and cartilage damage in RA [36], while IL-8 is the major chemoattractant for neutrophils, which play a critical role in initiating and maintaining inflammatory processes in the joint [37]. These cytokines can further stimulate the proliferative, invasive and inflammatory properties of RA synovial fibroblasts [1]. Additionally, the promoter region of MMP-9 possesses the functional enhancer element binding site for NF-κB, a transcription factor that is intimately involved in the regulation of the inflammatory responses in RA [38], which makes MMP-9 susceptible to induction by pro-inflammatory cytokines, particularly TNF-α [39]. Thus the elevated TNF-α in RA synovial fibroblasts stimulates MMP-9 and further boosts inflammation in RA synovial fibroblasts. Furthermore, our data indicate that not only can MMP-9 be stimulated by NF-κB, but it can reciprocally activate NF-κB, which forms an autocrine pathway to amplify and maintain the inflammation in RA synovial fibroblasts.

In contrast to the pro-inflammatory action of MMP-9, MMP-2 secreted by RA synovial fibroblasts exerts anti-inflammatory effects. This is supported by the evidence that suppressing MMP-2 increased TNF-α and IL-17 and activated NF-κB and the addition of rMMP-2 inhibited the activation of NF-κB by RA synovial fibroblasts. This function of MMP-2 is consistent with previous reports in other settings. For example, MMP-2 efficiently cleaves and inactivates monocyte chemoattractant protein 3 (MCP-3), resulting not only in blockage of the initiation of inflammatory response, but also complete abrogation of pre-existing inflammation in vivo [15]. MMP-2 protects against tissue damage and maintains gut barrier function in mice [33] and MMP-2 knockout mice develop more severe arthritis [15]. Hong et al. [16] demonstrated that MMP-2 is able to cleave and inactivate IL-17, a cytokine that enhances RA synovial fibroblast migration/invasion and inflammation [17]. Activated protein C, a natural anticoagulant, exerts its strong anti-inflammatory effects on RA synovial fibroblasts partly by promoting MMP-2 activity and inhibiting MMP-9 [5]. The effect of MMP-2 on TNF-α and IL-17 is likely via NF-κB as inhibition of this signalling molecule significantly reduced the production of TNF-α and IL-17 by RA synovial fibroblasts (Fig. 4).

Our data demonstrated the direct association of MMP-9 not only with RA synovial fibroblast-mediated inflammation, but also with cartilage degradation. Inhibition of MMP-9 resulted in decreased SGAG release from cartilage explants that were co-incubated with RA synovial fibroblasts (Fig. 6). MMP-9 degrades denatured collagens and activates other MMPs and cytokines [6]. Additionally, MMP-9 is thought to be involved in the activation of pro-MMP-13, a collagenase that has long been considered as the major enzyme involved in cartilage erosion in both OA and RA [40, 41]. Up-regulation of MMP-9, along with MMP-13 and MMP-14, coincides with the advancement of cartilage degeneration [42]. MMP-9 knockout mice show decreased cartilage matrix degradation [43].

**Fig. 6** Cartilage degradation by RA synovial fibroblasts in response to MMP-2 and/or MMP-9 inhibition/stimulation and inhibition of NF-κB, ERK, p38 and JNK

This effect of MMP-9 on RA synovial fibroblasts is likely via several different mechanisms. Previous researchers have shown that MMP-9 stimulates inflammation by cleavage of IL-8 to potentiate its activity by at least 10-fold [35] and segmentation of pro-IL-1β and pro-TNF-α into their active pro-inflammatory forms [13, 16].

SGAGs in culture medium from RA synovial fibroblasts transfected with MMP-2 (MMP2) and/or MMP-9 (MMP9) siRNA (10 nM). Cells were transfected with siRNA for 24 h and then trypsinized and re-seeded into 24 well plates. After overnight attachment, RA synovial fibroblasts were incubated with DMEM in the presence of OA cartilage with or without recombinant MMP-2 and/or MMP-9 (50 ng/ml) for another 24 h. Cartilage degradation was detected by measuring the SGAG released in culture medium using a DMMB assay. (B) SGAG in culture medium from RA synovial fibroblasts treated with MMP inhibitors for MMP-2 (tMMP2), MMP-9 (tMMP9), GM6001 (GM) (10 μM) or rMMP2/rMMP9 (50 ng/ml) for 24 h. (C) SGAG in culture medium from RA synovial fibroblasts treated with inhibitors of NF-κB, ERK, p38 and JNK (5 μM) for 24 h. Data are expressed as a percentage of control and are shown as mean (s.d.) (n = 5). *P < 0.05, **P < 0.01. MMP: matrix metalloproteinase; NF: nuclear factor; ERK: extracellular signal-regulated kinase; JNK: c-Jun NH2-terminal kinase; SGAGs: sulphated glycosaminoglycans; siRNA: small interfering RNA; DMMB: dimethylmethylene blue.
Conversely, in our study MMP-2 inhibited RA synovial fibroblast-mediated cartilage degradation, which is in agreement with a previous report showing that MMP-2 knockout mice display articular cartilage destruction and erosion of knee joints [44].

MMP-9-mediated cartilage destruction is likely to occur via activation of NF-κB and JNK, as inhibition of MMP-9 suppressed and rMMP-9 stimulated the activation of NF-κB and JNK (Fig. 5) and specific NF-κB and JNK inhibitors significantly prevented RA synovial fibroblast-mediated cartilage damage. Consistently, previous data show that NF-κB plays a fundamental role in the damage of articular tissues in RA [45] and JNK activation is involved in the signal transduction pathway leading to MMP-9 expression in RA synovial fibroblasts [46]. This also explains why inhibition of MMP-2 and MMP-9 together, which activated NF-κB, induced cartilage damage (Fig. 4). In contrast, rMMP-2 inhibits NF-κB activation, leading to decreased cartilage degradation (Fig. 6). Although inhibition of MMP-9 suppressed ERK activation, ERK-specific inhibition did not significantly affect cartilage damage, indicating that ERK is not involved in this process, which is in agreement with the finding that RA synovial fibroblast-mediated cartilage destruction does not depend on proliferation in RA [47]. Although p38 activation regulated RA synovial fibroblast-mediated cartilage degradation, we found no evidence that this MAP kinase was involved in MMP-2- or MMP-9-associated cartilage turnover by these cells.

MMP-2 and MMP-9 can degrade the ECM and release matrix-sequestered growth factors such as VEGF, TGF-β and fibroblast growth factors, thereby facilitating cell migration and invasion [48] and regulating cell survival and growth [49] in both normal and pathological conditions. These actions help explain how MMP-2 and MMP-9 promote RA synovial fibroblast survival, growth, migration and invasion. MMP-2 stimulated OA and RA synovial fibroblast viability equally, whereas MMP-9, which is not produced by OA synovial fibroblasts [5], enhanced cell survival only in RA synovial fibroblasts. Although MMP-2 and MMP-9 contributed equally to RA synovial fibroblast growth, MMP-9 was responsible for significantly increased survival, migration and invasion and reduced apoptosis compared with MMP-2 (Fig. 2), indicating that MMP-9 is more important for the survival and invasion of RA synovial fibroblasts. MMP-9-stimulated RA synovial fibroblast viability is likely to occur via activation of MAP kinase ERK and NF-κB, as inhibiting MMP-9 suppressed and MMP-9 stimulated the activation of ERK and NF-κB. In a similar manner, other studies using adenovirus-mediated delivery of siRNA in medulloblastoma cells have established a clear relationship between the loss of MMP-9 expression, apoptosis induction and activation of ERK and NF-κB [50, 51].

Together, these studies suggest that, despite their structural similarities, endogenous MMP-2 and MMP-9 exert opposing roles during RA synovial fibroblast-mediated cartilage degradation and inflammation. MMP-9 contributes to joint damage by increasing the survival, invasion and inflammatory cytokine release of RA synovial fibroblasts. Selective MMP-9 blockade may help prevent inflammation and cartilage damage in RA.

### Rheumatology key messages

- MMP-2 and MMP-9 contribute to the invasiveness of RA synovial fibroblasts, with MMP-9 having more effects.
- MMP-2 suppresses and MMP-9 promotes RA synovial fibroblast-mediated cartilage degradation and inflammation.

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### Supplementary data

Supplementary data are available at *Rheumatology* Online.

### References


