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TNF-α-mediated expression of membrane-type matrix metalloproteinase in rheumatoid synovial fibroblasts

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SUMMARY

Degradation of the extracellular matrix plays an important role in rheumatoid articular destruction. Rheumatoid synovial fibroblasts secrete a large amount of matrix-degrading metalloproteinases (MMPs), which initiate tissue damage by proteolytic degradation of collagens and proteoglycans. Cytokines, such as interleukin-1α, -1β or tumour necrosis factor (TNF)-α, are potent inducers of MMPs in rheumatoid synovial fibroblasts. MMPs are synthesized and secreted as latent pro-enzymes and their activation is achieved by proteolytic cleavage of the propeptide domain at the N-terminus of the molecule. Thus, the interaction of the pro-enzymes with specific activators determines the enzymatic activity in the extracellular space. In the present study, we identified a novel mechanism for the activation of pro-MMP-2, which can be achieved through the interaction of the inflammatory cytokine, TNF-α, with synovial fibroblasts. Although MMP-2 is constitutively secreted by synovial fibroblasts as a pro-enzyme, stimulation of fibroblasts by TNF-α-induced secretion of MMP-2 in an active form. In support of this result, TNF-α stimulation-induced membrane-type matrix metalloproteinase (MT-MMP), a newly identified MMP-2-specific activator, on synovial fibroblasts. Cycloheximide analysis demonstrated that protein synthesis may be required for TNF-α-mediated MT-MMP expression on synovial fibroblasts. Our results suggest that TNF-α induces MMP-2 activation in part by up-regulating MT-MMP expression, thus representing a new mechanism for cytokine-mediated articular destruction in rheumatoid arthritis (RA).

INTRODUCTION

Rheumatoid synovial fibroblasts produce a number of matrix metalloproteinases (MMPs) including MMP-1, 2, 3, 9.[1–3] High concentrations of these MMPs are present in synovial fluid of patients with rheumatoid arthritis (RA)[4–6] and cytokines produced by synovial inflammatory cells are thought to induce the secretion of MMPs from rheumatoid synovial cells.[3,7,8] MMPs are a family of enzymes capable of degrading most of the constituent macromolecules of the extracellular matrix.[9] MMP-2, like other MMPs,[10–12] is secreted in a latent zymogen form (pro-enzyme) requiring processing for the conversion to the active form.[13] The process of pro-enzyme activation is, therefore, a critical event in the regulation of MMP-2 enzymatic activity and plays an important role in MMP-2-mediated extracellular matrix (ECM) degradation in the rheumatoid arthritis (RA) synovium.[3,14]

MMP-2 is unique since it can be activated by cell surface-associated activator.[15,16] Previous reports suggested that the putative MMP-2 activator is located on the cell surface and is inducible by factors such as concanavalin A (Con A) or 12-*O*-Tetradecanoyl phorbol-13-acetate (TPA).[17,18] Recently, a new membrane-type matrix metalloproteinase (MT-MMP) that induces a specific activation of pro-MMP-2 was identified.[19] More recent studies indicated that the expression of MT-MMP is enhanced by MMP-2-activating factors such as Con A.[20]

To identify the interaction between the secreted pro-MMP-2 and its specific activator in the rheumatoid synovium, we examined the effects of tumour necrosis factor-α (TNF-α) on MMP-2 activation. Our results indicated that stimulation by TNF-α augments the secretion of the active form of MMP-2 from rheumatoid synovial fibroblasts. Furthermore, we analysed the expression of the newly identified MT-MMP on TNF-α-stimulated synovial fibroblasts. Our data suggest a novel mechanism for cytokine-induced pro-MMP-2 activation that may induce ECM degradation in the rheumatoid joint.

MATERIALS AND METHODS

Materials

Mouse anti-human MMP-2 monoclonal antibody was purchased from Oncogene Science Inc. (Cambridge, MA). This antibody reacts with the latent and active forms of MMP-2. Mouse anti-human MT-MMP was purchased from Fuji Chemical (Takaoka, Japan). Human recombinant TNF-α was kindly provided by Dainihon Chemical Co. (Osaka, Japan).

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Preparation of synovial cells

The protocol was approved by the local ethics committees and a signed consent form was obtained. Synovial tissue samples were obtained from patients with RA ($n = 7$, two males five females, the mean age, 45·3 ± 22·6) during synovectomy. The synovial membranes were minced aseptically, then dissociated enzymatically with collagenase (4·0 mg/ml, Sigma Chemicals Co., St Louis, MO) in RPMI-1640 for 4 hr at 37°. The obtained cells were plated in culture dishes and allowed to adhere. To eliminate non-adherent cells from the synovial cell preparations, the plated cells were cultured for 18 hr with RPMI-1640, supplemented with 10% fetal calf serum (FCS) at 37° in humidified 5% CO_2 in air. Cells were then washed extensively with phosphate-buffered saline solution (PBS). Adhering synovial cells were removed by adding trypsine-EDTA and washed with PBS containing 2% FCS. The collected synovial cells were used at the third or fourth passage for subsequent experiments. Synovial cell preparations were less than 1% reactive with monoclonal antibodies CD3, CD20, CD68 (Coulter Immunology, FL) and anti-human von Willebrand factor (Immunotech, Marseille, France), which respectively, define an antigen on mature lymphocytes, monocytes/macrophage, and vascular endothelial cells. Cells were plated in a six-well culture plate (Coster, Cambridge, MA) for 24 hr, then washed three times with PBS before the addition of serum-free RPMI-1640 medium supplemented with various concentrations of TNF-α. The conditioned medium was collected after 48 hr of incubation, centrifuged (3500 ***g*** for 5 min) to clarify debris and analysed by immunoblot.

Gelatin zymography

The culture media were incubated at 37° for 20 min in sodium dodecyl sulphate (SDS) sample buffer without reducing agent and then electrophoresed on 8% polyacrylamide gels containing 1% gelatin at 4°. After electrophoresis, the gels were washed in 2·5% Triton-X 100 to remove SDS, and incubated with 50 mM Tris-HCl buffer (pH 7·5) containing 0·15 M NaCl, 10 mM $CaCl_2$ and 0·02% NaN_3 for 16 hr at 37° and stained with 0·1% Coomassie Brilliant Blue R250.

Immunoblot analysis

Immunoblot analysis for MT-MMP was performed according to the method of Yu *et al.*[20] Synovial monolayers were rinsed with Tris-buffered saline (10 mM Tris-HCl pH 7·5, 150 mM NaCl), scraped in ice-cold Tris-buffered saline and sonicated by ultrasound sonicator (Wakenyaku Co. Ltd, Tokyo, Japan) on ice in the presence of proteinase inhibitors (in mM: 0·1 aprotinin, 1·0 leupeptin, 1·0 EDTA and 1·0 pepstain). Cellular lysate were saved after centrifugation and the protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). An identical quantity of proteins (40 μg) of each lysate was subjected to 8% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). In addition, synovial fibroblast-conditioned media were dissolved on SDS–PAGE, and proteins were transferred to nitrocellulose membrane. The filters were blocked for 1·5 hr using 5% non-fat dried milk in Tris-buffered solution (TBS) (50 mM Tris, 0·15 M NaCl, pH 7·5) containing 0·1% Tween 20, washed with TBS and incubated at room temperature for 2 hr in a 1:100 dilution of mouse anti-MMP-2 monoclonal antibody (mAb) or a 1:250 dilution of mouse anti-MT-MMP mAb. Filters were later washed with TBS and incubated with 1:1000 dilution of donkey anti-mouse immunoglobulin G (IgG) antibodies, coupled with horseradish peroxidase. The enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL) was used for detection. Filters were subsequently exposed to the film for 15 seconds and the latter was processed.

RESULTS

Synovial fibroblasts isolated from RA patients were seeded in RPMI-1640 medium supplemented with FCS for 48 hr before switching serum-free medium. Synovial fibroblasts were incubated for 48 hr in the presence or absence of TNF-α (10 U/ml, 100 U/ml). Conditioned media were collected and electrophoresed on gelatin substrate gels to analyse the gelatin-degrading activity. Gelatin zymography of media from TNF-α-stimulated fibroblasts demonstrated an induction of gelatinase activity that corresponded to an electrophoretic mobility of 72 000 MW and 66 000 MW (Fig. 1a).

Activation of 72 000 MW progelatinase A (MMP-2) involves autocatalytic cleavage to yield to an active form of MMP-2. 72 000 MW and 66 000 MW gelatinolytic bands detected in TNF-α-stimulated synovial fibroblast-conditioned media should be pro-MMP-2 (gelatinase A) and active forms of MMP-2. To confirm whether pro-MMP-2 secreted from TNF-α-stimulated synovial fibroblasts are converted to active forms of MMP-2, western analysis was carried out using anti-MMP-2 mAb that recognizes pro and active forms of MMP-2. Pro-MMP-2, which migrates as a 72 000 MW band, was detected in the media conditioned by non-treated synovial fibroblasts. Incubation of synovial fibroblasts with TNF-α increased the secretion of 72 000 MW pro-MMP-2. Furthermore, the active form of MMP-2, which migrates to 66 000 MW upon reduction, was demonstrated in the conditioned media (Fig. 1b). These data demonstrated that activation of MMP-2 occurred in RA synovial fibroblast cultures when the cells were stimulated by the inflammatory cytokine, TNF-α.

It has been demonstrated that activation of pro-MMP-2 is achieved by a membrane activator. Recently, Sato *et al.*[19] cloned the cDNA encoding a new MT-MMP that induces the specific activation of pro-MMP-2. Therefore, we examined whether synovial fibroblasts express MT-MMP using western analysis. MT-MMP was constitutively expressed in synovial fibroblasts under baseline conditions. Furthermore, we found that stimulation of TNF-α facilitated MT-MMP expression on synovial fibroblasts (Fig. 2a) in a dose-dependent manner (Fig. 2b). We conducted a dose-response study using cycloheximide, an inhibitor of protein synthesis, on TNF-α-induced expression of MT-MMP in synovial fibroblasts. Synovial fibroblasts were incubated with 100 U/ml TNF-α in the presence of various concentrations of cycloheximide. Cycloheximide treatment inhibited TNF-α-induced MT-MMP expression on synovial fibroblasts in a dose-dependent manner (Fig. 2c). The data suggested that protein synthesis is required for TNF-α-induced MT-MMP expression.

Finally, we examined the ability of TNF-α-stimulated synovial fibroblasts to secrete MT-MMP in culture medium. Conditioning media from TNF-α-stimulated or -non-stimulated synovial fibroblasts were analysed by western analysis using anti-MT-MMP mAb. MT-MMP was not detected in the culture media of control synovial fibroblasts. On the other hand, we detected a 54 000 MW band, which reacted with anti-MT-MMP mAb, in TNF-α-stimulated synovial fibroblasts conditioned media (Fig. 3, lane 2). However, this band was not the size of the full-length MT-MMP (64 000 MW) as demonstrated in synovial fibroblasts cell lysates (Fig. 3, lane 3). Although the exact magnitude of

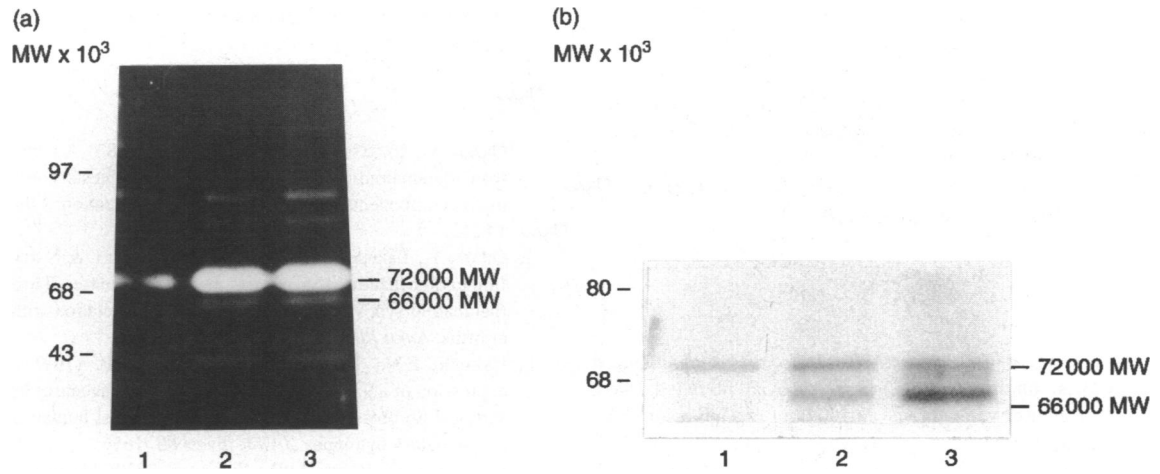

Figure 1. MMP-2 secretion from TNF-α-stimulated rheumatoid synovial fibroblasts. (a) Gelatine zymography. Theconditioned media were electrophoresed on a gelatine substrate gel and stained with Coomassie brilliant blue R-250. Zones of clearing represent gelatinolytic activity. Prestained molecular weight markers are in the far left lane. Lane 1: control rheumatoid synovial cells; lane 2: stimulated cells with 10 U/ml of TNF-α; lane 3: 100 U/ml of TNF-α for 48 hr. (b) Immunoblot analysis. The conditioned media were electrophoresed on SDS–PAGE, transferred to filter and subjected to immunoblot analysis using anti-MMP-2 mAb. The antibody identified a band at 72 000 MW (pro-MMP-2) and a band at 66 000 MW (active form of MMP-2) in media from TNF-α-stimulated rheumatoid synovial fibroblasts. Lane 1: control rheumatoid synovial cells; lane 2: stimulated cells with 10 U/ml of TNF-α; lane 3: 100 U/ml of TNF-α for 48 hr. Experiments were repeated at least three times.

degradation occurring in secreted MT-MMP was not evaluated, this protein segment may represent a truncated form of MT-MMP.

DISCUSSION

Although the precise mechanism of articular destruction in RA is not completely understood, it has been reported that the rheumatoid synovial fluid contains various proteinases that may contribute to the cartilage damage.[21] Furthermore, elevated levels of MMPs, which induce extracellular matrix degradation, have been detected in joint fluid and synovium of patients with RA.[4–6] In a series of studies, Dayer *et al.*[7,8] demonstrated that inflammatory cytokines, such as interleukin-1 (IL-1) and TNF-α, contribute to tissue destruction indirectly by stimulating MMPs production from rheumatoid synovial fibroblasts.

MMPs are secreted in a latent inactive form and their conversion to the active form can be accomplished by proteolytic enzymes or other MMPs.[10–13] The Mr 72 000 gelatinase A (pro-MMP-2) is a member of MMPs capable of degrading several connective tissue components including collagens. Several studies have shown that MMP-2 is secreted from rheumatoid synovial fibroblasts.[3,13] However, the process responsible for the activation of pro-MMP-2, which may directly contribute to the degradation of rheumatoid cartilage components, is not completely understood.

In the present study, we examined the influence of the inflammatory cytokine, TNF-α, on activation of pro-MMP-2

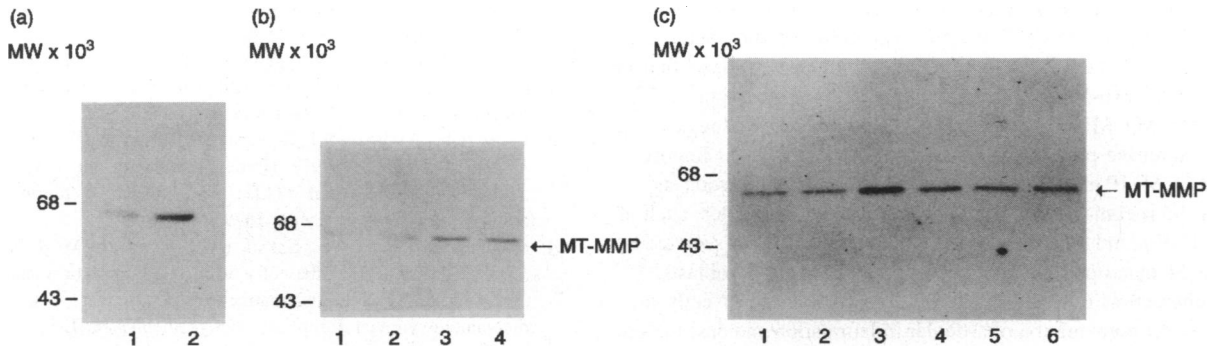

Figure 2. Expression of membrane-type matrix metalloproteinase (MT-MMP) in rheumatoid synovial fibroblasts. (a) Rheumatoid synovial fibroblasts were incubated with (lane 2) or without (lane 1) 100 U/ml of TNF-α for 48 hr. Cellular lysates were analysed by immunoblot using anti-MT-MMP mAb. (b) Rheumatoid synovial cells were incubated with TNF-α at a concentration of 0 (lane 1), 1·0 (lane 2) 10 (lane 3), or 100 U/ml (lane 4), for 48 hr. Cellular lysates were analysed by immunoblot using anti-MT-MMP mAb. (c) Rheumatoid synovial fibroblasts were incubated with 100 U/ml of TNF-α, in the presence of various concentrations of cycloheximide for 48 hr. Cellular lysates were analysed by immunoblot using anti-MT-MMP mAb. Lane 1: without TNF-α and cycloheximide; lane 2: 10 μg/ml of cycloheximide only; lanes 3–6: TNF-α (100 U/ml); lane 3: TNF-α only; lane 4: TNF-α and 1 μg/ml of cycloheximide; lane 5: TNF-α and 10 μg/ml of cycloheximide; lane 6: TNF-α and 50 μg/ml of cycloheximide. Experiments were repeated at least three times.

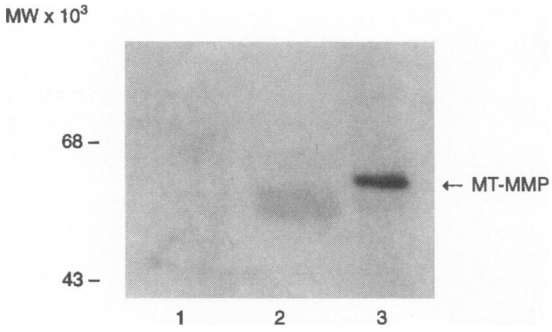

Figure 3. No secretion ofo full-length MT-MMP from TNF-α-stimulated rheumatoid synovial fibroblasts. Rheumatoid synovial fibroblasts were incubated with (lane 2) or without (lane 1) 100 U/ml of TNF-α for 48 hr. Conditioned media were analysed by immunoblot using anti-MT-MMP mAb. Lane 3 indicates the positive control for MT-MMP (cellular lysates). Experiments were repeated at least three times.

secreted from rheumatoid synovial fibroblasts. Our results demonstrate that MMP-2 is constitutively produced by rheumatoid synovial fibroblasts in a latent form, and that stimulation by TNF-α induced the conversion of pro-MMP-2 to the active form of MMP-2.

Sato *et al.*[19] recently identified a 63 000 MW new membrane type of MT-MMP and the expression of MT-MMP induced a specific activation of pro-MMP-2. We examined this newly identified MT-MMP expression in synovial fibroblasts. Our results demonstrated that MT-MMP is constitutively expressed in synovial fibroblasts and that the expression was increased by TNF-α stimulation. In addition, analysis of cycloheximide studies suggests that protein synthesis may be involved in TNF-α-induced MT-MMP expression. In this regard, previous reports suggested that a putative MMP-2 activator is localized to the cell surface and is up-regulated by Con A or TPA.[17,18] Further studies by Yu *et al.*[20] demonstrated that Con A treatment enhanced the expression of MT-MMP in human breast cancer cells. They hypothesized that activation of exogenous MMP-2 by MT-MMP on the malignant cells is critical for tumour cell invasion and metastasis.[19] Our data suggest a novel role for TNF-α in inducing MMP-2 activation, possibly mediated by MT-MMP, in inflammatory joint diseases.

Based on these results, we propose a potential role for activation of pro-MMP-2 in the destruction of the rheumatoid joint. In our model, TNF-α stimulates synovial fibroblasts that in turn convert pro-MMP-2 to the active form by expressing MMP-2 activator, MT-MMP, on the cell surface. Our results suggest that the constitutive production of pro-MMP-2 may be less important than MT-MMP expression on rheumatoid synovial fibroblasts.

In the rheumatoid synovium, inflammatory cytokines, such as IL-1, TNF-α and transforming growth factor (TGF), are secreted by activated macrophages, monocytes and synovial fibroblasts.[22,23] The interaction of these cytokines and inflammatory cells may result in the perpetuation of articular inflammation and destruction. TNF-α has been considered the primary candidate among the different cytokines responsible for synovitis in RA.[24] In support of this role, Elliot and coworkers[25,26] demonstrated recently that administration of anti-TNF-α monoclonal antibody abates the joint inflammation in RA patients. In this report, we suggest that this cytokine may confer the synovial fibroblasts enhanced ability to degrade cartilage matrix components by activating MMP-2. Furthermore, our results implicate the TNF-α-mediated MT-MMP expression on synovial fibroblasts in a critical event in the process of MMP-2 activation.

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