EFFECTS OF INFLAMMATORY MEDIATORS ON MATRIX METALLOPROTEINASE-2 EXPRESSION BY RHEUMATOID ARTHRITIS SYNOVIAL FIBROBLASTS

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DEDICATION

To my parents.

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ABBREVIATIONS

Abbreviation	Full description
AgIA	antigen induced arthritis
BCA	bicinchoninic acid
COX-2	cyclooxygenase-2
DAPI	4',6-diamidino-2-phenylindole-dihydrochloride
DMSO	dimethyl sulfoxide
EC	endothelial cell
ECL	enhanced chemiluminescence
ELISA	enzyme-linked immunoadsorbent assay
ENA-78	epithelial neutrophil-activating peptide-78
ERK	extracellular-signal-regulated kinase
FBS	fetal bovine serum
Gro-α	Growth-regulated oncogene alpha
IFN-γ	interferon-gamma
Ig	immunoglobulin
IHC	immunohistochemistry
IL-1β	interleukin-1beta
Jak	janus kinase
JNK	c-jun N-terminal kinase
MAPK	mitogen activated protein kinase
mBSA	methylated bovine serum albumin
MIF	macrophage migration inhibitory factor
MIF -/-	MIF gene deficient
MIP-1	macrophage inflammatory protein
MMPs	matrix metalloproteinases
MT-MMP	membrane type matrix metalloproteinase
NF-κB	Nuclear factor-kappa B
NS	non-stimulated
PBS	Dulbecco's phosphate buffered saline
PDTC	pyrrolidine dithiocarbamate
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
RA	rheumatoid arthritis
RANTES	regulated upon activation normal T cell expressed and secreted
SCID	severe combined immunodeficient
SDS	sodium dodecyl sulphate
STAT3	signal transducer and activator of transcription 3
TBST	tris-buffered saline tween buffer
TIMP	tissue inhibitor of metalloproteins
TNF- α	tumor necrosis factor-alpha
WT	wild type
ZIA	zymosan induced arthritis

1. INTRODUCTION

1.1 Rheumatoid arthritis synovial fibroblasts

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial hyperplasia and invasion and destruction of bone and cartilage. RA synovial fibroblasts (also termed fibroblast-like synoviocytes or type B synoviocytes), together with synovial macrophages, are the two leading cell types in the terminal layer of the hyperplastic synovial tissue that invades and degrades adjacent cartilage and bone[1,2].

In non-diseased tissue, the physiological function of synovial fibroblasts is to provide the joint cavity and the adjacent cartilage with nutritive plasma proteins and lubricating molecules such as hyaluronic acid. Synovial fibroblasts are also involved in continuous matrix remodeling by producing matrix components such as collagen and hyaluronan as well as a variety of matrix-degrading enzymes[1].

Although there is sufficient information to support the role of synovial fibroblasts as a major contributor to joint degradation, their functional role in modulating the pathogenic inflammatory and autoimmune responses in RA joints remains poorly defined. In response to extracellular factors, synovial fibroblasts themselves release a plethora of effector molecules interacting with a variety of cells and promoting matrix degradation[1]. First, synovial fibroblasts instigate leukocyte attraction and homing through the expression of chemokines like macrophage inflammatory protein 1 (MIP-1)/CCL3, RANTES (regulated upon activation, normal T-cell expressed and secreted)/CCL5 and interleukin-8 (IL-8)/CXCL8. (See chemokine nomenclature later.)

In addition, synovial fibroblasts can also support myeloid and lymphoid cell growth via the secretion of various colony stimulating factors, as well as their own growth via the production of platelet derived growth factor. However, the most classical effector function of RA synovial fibroblasts is their capacity to interact with the extracellular matrix and cause its degradation via the production of matrix metalloproteinases (MMPs) and cathepsins. Interestingly, synovial fibroblasts may also be able to dampen the immune response via the production of transforming growth factor-β, soluble tumor necrosis factor-α (TNF-α) receptors and IL-10, as well as extracellular matrix degradation by tissue inhibitor of metalloproteins (TIMP).[1] The aggressive potential of synovial fibroblasts is convincingly demonstrated in the severe combined immunodeficient (SCID) mouse model of cartilage destruction, in which implanted human RA synovial fibroblasts degrade human co-implanted cartilage in the absence of inflammatory cells.[2]

1.2 Matrix metalloproteinases

MMPs are a large family of proteolytic enzymes responsible for degradation of extracellular matrix components and are thought to play a crucial role in the RA joint destruction.[3] In RA, angiogenesis is thought to be a key event in the expansion of the synovial lining of the joints. Angiogenesis requires proteolysis of the extracellular matrix, proliferation, and migration of endothelial cells, as well as synthesis of new matrix components. MMPs play a crucial role in the angiogenic process. The evidence for this conclusion is that MMP inhibitors block angiogenic responses both *in vitro* and *in vivo*.[7-9]

1.2.1 Classification of MMPs

MMPs are classified into 5 subgroups based on structural domains and substrate specificity:

1. collagenases such as interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8), and collagenase-3 (MMP-13).
2. gelatinases including gelatinase A (MMP-2) and gelatinase B (MMP-9).
3. stromelysins such as stromelysin-1 (MMP-3) and stromelysin-2 (MMP-10).
4. membrane type MMPs (MT-MMP) including MT1-, MT2-, MT3-, MT4-, MT5- and MT6-MMP.
5. other MMPs such as matrilysin, stromelysin 3, metalloelastase, enamelysin and MMP-19.

1.2.2 MMP structure

MMP structure consists of a signaling peptide at the N-terminal region, which allows for secretion of the enzyme into the endoplasmic reticulum and transport out of the cell. Adjacent to the signal peptide is a hydrophobic propeptide domain that shields the neighboring catalytic domain, which contains a Zn^{2+} ion. The catalytic domain of MMP-2 and MMP-9 is unique from that of other MMPs in that it contains three fibronectin type II-like domains which form a collagen-binding domain, allowing for the binding and subsequent cleavage of type IV collagen or denatured collagen.[10] The haemopexin-like C-terminal region is connected to the catalytic domain by a flexible hinge region and can allow for the binding of other proteins, which may serve to alter the activity of the MMP.

1.2.3 MMP-2 activation

Most MMPs are secreted as latent proenzymes and their activation requires proteolytic degradation of the propeptide domain. This activation occurs extracellularly and is often mediated by activated MMPs.[11] Activation of proMMPs requires the dissociation of the binding between the cysteinyl sulphydryl in the propeptide domain and the catalytic Zn^{2+} ion. This critical cysteine residue and the Zn^{2+} catalytic domain are common to all MMPs. The so-called cysteine switch is believed to be a common mechanism of activation of MMPs.[12] Cell surface activation of 72 kDa MMP-2 occurs as a result of interaction with MT1-MMP and TIMP-2. A second molecule of MT1-MMP then interacts with the MMP-2/TIMP-2/MT1-MMP complex and cleaves the propeptide domain from MMP-2 resulting in 64 kDa active MMP-2.[13] The binding of other proteins to the haemopexin domain of MMP-2 has been shown to expedite its activation. The interaction of the integrin $\alpha_V\beta_3$ with MMP-2 facilitates this complex process of MMP-2 activation.[14,15] In addition, other proteins such as heparin, thrombin, factor Xa, proteinase-3, neutrophil elastase and cathepsin G also play important roles in the activation of MMP-2, although the mechanism by which they act is not yet fully elucidated.[16-20]

1.2.4 MMP-2 functions

Despite distinct classification, the role of each individual MMP in a specific process such as RA is not clear yet. However, MMPs are thought to participate in extracellular matrix degradation in several pathological conditions including bone remodeling, atherosclerosis, apoptosis, angiogenesis, tumor invasion and RA.[4, 21-28] In addition, MMPs are associated with various rheumatologic inflammatory conditions such as psoriatic arthritis, lupus, gout, osteoarthritis and RA (Table 1.)

MMP-2 degrades gelatin, collagen type I, II, III, IV, V, VII, X, fibronectin, elastin and laminin.[29] MMP-2 has been shown to be secreted by fibroblasts, keratinocytes, epithelial cells, monocytes and osteoblasts.[30]

It has been suggested previously that MMP-2 plays an important role in RA. RA patients with radiographic erosions have significantly higher levels of active MMP-2 in their synovial tissues than patients with no erosions, suggesting that MMP-2 plays a crucial role in joint destruction.[31] In addition, MMP-2 has been previously linked to invasion of RA synovial fibroblasts[32,33] and implicated in angiogenesis.[4,5] Elevated MMP levels (MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9 and MMP-13) are detected in RA compared to osteoarthritic synovial fluid.[34] In the RA synovium, MMP-2 is expressed in the lining and sublining layers as well as at the synovial membrane-cartilage interface.[35,36]

Table 1 Inflammatory disorders associated with MMPs (Adapted from C Nathan, Nature 2002; 420:846-852)

Alzheimer's disease	Psoriasis
Asthma	Rheumatoid arthritis
Atherosclerosis	Sarcoidosis
Atopic dermatitis	Systemic lupus erythematosus
Bullous pemphigoid	Type 1 diabetes mellitus
Chronic obstructive pulmonary disease	Ulcerative colitis
Gout	Helicobacter pylori infection
Inflammatory bowel disease	Hepatitis C
Ischaemia reperfusion injury	Neisserial and pneumococcal meningitis
Multiple sclerosis	Tuberculosis
Osteoarthritis	

1.3 Macrophage migration inhibitory factor

Macrophage migration inhibitory factor (MIF) was originally identified as a protein derived from T lymphocytes.[37,38] MIF is a proinflammatory cytokine produced by macrophages in response to inflammatory stimuli such as TNF-α or interferon-gamma (IFN-γ).[39] MIF induces the production of a large number of proinflammatory mediators, such as TNF-α, IFN-γ, interleukin-1β (IL-1β), IL-6, IL-8, nitric oxide and cyclooxygenase-2 (COX-2).[39-42] Recently, MIF has been implicated in angiogenesis[43,44] as well as in the pathogenesis of RA.[45] Several independent studies described MIF enhancing angiogenesis and playing a role in tumor neovascularization.[46,47] In type II collagen-induced arthritis, a murine model of RA, neutralizing anti-MIF antibody delayed the onset and decreased the frequency of arthritis.[45] Moreover, MIF gene deficient (MIF -/-) mice exhibited significantly less synovial inflammation than wild type (WT) mice after arthritis induction with type II collagen.[48]

1.4. Epigallocatechin-3-gallate

Green tea (*Camellia sinensis*) is one of the most commonly consumed beverages in the world, with no significant side effects.[49] Extensive studies in several animal models in the past two decades have verified the antioxidant, antiinflammatory, and antioncogenic properties of a polyphenolic mixture derived from green tea.[50] A majority of pharmacologic properties of green tea are mimicked by its active constituent, epigallocatechin-3-gallate (EGCG).[51] Previous studies using human chondrocytes derived from osteoarthritic cartilage showed EGCG to be an effective inhibitor of the production of catabolic mediators, such as nitric oxide (NO) and prostaglandin E_2 (PGE_2), by transcriptional and translational regulation.[52-54] EGCG significantly inhibited the expression and activities of MMP-1 and MMP-13 in human chondrocytes at a physiologically achievable dose.[55]

Nonetheless, significant gaps remain in our understanding of the mechanism of action of EGCG in RA.

1.5 Chemokines

Chemokines, currently numbering more than 50, are chemotactic cytokines that are important in recruitment of leukocytes and in angiogenesis. They exert chemotactic activity toward a variety of cell types.[56-58] (Szekanecz et al. Chemokines and angiogenesis in rheumatoid arthritis, Front Biosci, in press). Chemokines have been classified into 4 supergene families based on the location of cysteine residues (Table 2). The 4 groups are CXC, CC, C, and CX_3C chemokines. A relatively new classification system was introduced in 2000, in which chemokines are considered as chemokine ligands, and each chemokine has been assigned a designation of CXCL, CCL, XCL, or CX_3CL1, respectively.[59, 60]
At least 18 human seven-transmembrane-domain chemokine receptors have been identified. Interaction of chemokines with these specific G-protein coupled receptors on target cells produces their biological effects, which includes promoting cell adhesions, invasion, mobilization, interactions with the extracellular matrix.[61]

In RA, chemokines mediate the migration of inflammatory leukocytes into the synovium. Among the four known chemokine families, CXC, CC chemokines and fractalkine/CX_3CL1 seem to be of outstanding importance in this process. Angiogenesis is also important during the perpetuation of inflammation underlying rheumatoid arthritis. CXC chemokines containing the ELR amino acid motif such as epithelial-neutrophil activating protein-78 (ENA-78)/CXCL5 and growth-regulated oncogene product alpha (gro-α)/CXCL1 promote angiogenesis.[62] ENA-78/CXCL5 is a chemotactic factor for neutrophils and is angiogenic.[63-66] Amounts of ENA-78/CXCL5 produced by RA synovial fibroblasts are

much higher than those released by osteoarthritic synovial fibroblasts.[67] RA synovial fibroblasts constitutively produce ENA-78/CXCL5, and this production is augmented by IL-1, TNFα, or IL-18.[67,68] ENA-78/CXCL5 accounts for a portion of the angiogenic activity observed in RA synovium.[65] Gro-α/CXCL1 is a potent neutrophil chemoattractant found in RA synovial fibroblasts and synovium.[69,70] Gro-α/CXCL1 is inducible by TNF-α and IL-1β in RA synovial fibroblasts.[70,71] Furthermore, gro-α/CXCL1 induces expression of interstitial collagens by RA fibroblasts.[72] RANTES/CCL5 chemoattracts lymphocytes and monocytes, as well as other cell types.[73,74] RA synovial fibroblasts produce RANTES/CCL5 mRNA upon stimulation by TNF-α, IL-1 or IFN-γ.[74-76] RANTES/CCL5 is involved in cytokine networks by inducing RA synovial fibroblasts to produce IL-8/CXCL8 and IL-6.[77]

Another important function of chemokines is to mobilize endothelial progenitor cells to the RA synovium and promote vasculogenesis. (Pakozdi A et al. Endothelial progenitor cells in arthritis-associated vasculogenesis and atherosclerosis, Joint Bone Spine, in press)

Chemokines also facilitate a unique mechanism of tissue invasion in the diseased joint by enhancing RA synovial fibroblast proliferation and MMP up-regulation.[78] Regulation of MMP-2 activity is of therapeutic value since it plays a predominant role in processes such as angiogenesis, inflammation, and tissue invasion.[79]

Table 2 CXC, C, CX3C, and CC chemokine/receptor families. (Adapted/modified from R Thorpe, J Immunol Methods 2002;262:1–3)

Systemic name (ligand)	Traditional name (ligand)	Systemic name (receptor)
CXC Chemokine/receptor family		
CXCL1	Gro-α/MGSA-α	CXCR2 > CXCR1
CXCL2	Gro-β/MGSA-β	CXCR2
CXCL3	Gro-γ/MGSA-γ	CXCR2
CXCL4	PF4	Unknown
CXCL5	ENA-78	CXCR2
CXCL6	GCP-2	CXCR1, CXCR2
CXCL7	NAP-2	CXCR2
CXCL8	IL-8	CXCR1, CXCR2
CXCL9	Mig	CXCR3
CXCL10	IP-10	CXCR3
CXCL11	I-TAC	CXCR3
CXCL12	SDF-1α/β	CXCR4
CXCL13	BCL	CXCR5
CXCL14	BRAK/bolekine	Unknown
(CXCL15)	Unknown	Unknown
CXCL16		CXCR6
C Chemokine/receptor family		
XCL1	Lymphotactin/SCM-1α/ATAC	XCR1
XCL2	SCM-1β	XCR1
CX3C Chemokine/receptor family		
CX3CL1	Fractalkine	CX3CR1
CC Chemokine/receptor family		
CCL1	I-309	CCR8
CCL2	MCP-1/MCAF/TDCF	CCR2
CCL3	MIP-1α/LD78α	CCR1, CCR5
CCL3L1	LD78β	CCR1, CCR5
CCL4	MIP-1β	CCR5
CCL5	RANTES	CCR1, CCR3, CCR5
(CCL6)	Unknown	Unknown
CCL7	MCP-3	CCR1, CCR2, CCR3
CCL8	MCP-2	CCR3, CCR5
(CCL9/CCL10)	Unknown	CCR1
CCL11	Eotaxin	CCR3
(CCL12)	Unknown	CCR2
CCL13	MCP-4	CCR2, CCR3
CCL14	HCC-1	CCR1, CCR5
CCL15	HCC-2/Lkn-1/MIP-1δ	CCR1, CCR3
CCL16	HCC-4/LEC/LCC-1	CCR1, CCR2
CCL17	TARC	CCR4
CCL18	DC-CK1/PARC/AMAC-1	Unknown
CCL19	MIP-3β/ELC/exodus-3	CCR7
CCL20	MIP-3α/LARC/exodus-1	CCR6
CCL21	6-chemokine/SLC/exodus-2	CCR7
CCL22	MDC/STCP-1	CCR4
CCL23	MPIF-1/CKβ8/CKb8-1	CCR1
CCL24	Eotaxin-2/MPIF-2	CCR3
CCL25	TECK	CCR9
CCL26	Eotaxin-3	CCR3
CCL27	CTACK/ILC	CCR10
CCL28	MEC	CCR3/CCR10

2. AIMS

In the present study our goal was to further evaluate MMP-2 expression in the RA synovium. Hence, we aimed:

1. To determine the effect of MIF on RA synovial fibroblast MMP-2 expression *in vitro*
2. To define signal transduction pathways playing a role in MIF-induced RA synovial fibroblast MMP-2 expression *in vitro*
3. To investigate MMP-2 expression in inflammatory arthritis in MIF -/- and WT mice *in vivo*
4. To elucidate the effect of chemokines on RA synovial fibroblast MMP-2 expression *in vitro*
5. To investigate whether EGCG inhibits cytokine and chemokine-induced RA synovial fibroblast MMP-2 expression *in vitro.*

3. MATERIALS AND METHODS

3.1 Antibodies and reagents

Recombinant human MIF, recombinant human TNF-α (ED_{50} 0.02-0.05 ng/ml), IL-1β, recombinant human MMP-2 and MMP-9 were purchased from R&D Systems (Minneapolis, MN). EGCG was purchased from Sigma-Aldrich (St. Louis, MO). The following specific inhibitors were obtained from Calbiochem (La Jolla, CA): phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002; mitogen activated protein kinase extracellular-signal-regulated kinase (MAPK/ERK, MEK) inhibitor, PD98059; Src inhibitor PP2; janus kinase (Jak) inhibitor, AG490; nuclear factor-kappa B (NF-κB) inhibitor, pyrrolidine dithiocarbamate (PDTC); p38 mitogen-activated protein kinase inhibitor, SB203580; JNK inhibitor II; PKC inhibitor, Ro-31-8425; specific PKC$\tilde{\alpha}\beta$ inhibitor, Gö 6976; PKCδ inhibitor, rottlerin and signal transducer and activator of transcription 3 (STAT3) inhibitor peptide. The G-protein inhibitor pertussis toxin was purchased from Sigma. Inhibitors were dissolved in distilled water or dimethyl sulfoxide (DMSO) according to the manufacturer's instructions. Rabbit anti-human phospho-specific antibodies directed against phosphorylated forms of PKC (pan) (Thr514), PKC$\tilde{\alpha}_{I}\beta_{II}$ (Thr638/641), PKCδ (Tyr311), PKCδ (Thr505), SAPK/JNK (Thr183/Tyr185) and c-Jun (Ser63) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Rabbit anti-human phospho-PKCε (Ser729) was purchased from Upstate (Lake Placid, NY). Mouse anti-human MMP-2 was purchased from R&D Systems, rabbit anti-mouse MMP-2 was obtained from Novus Biologicals (Littleton, CO). Goat anti-rabbit alkaline phosphatase conjugated antibody and rabbit anti-human actin antibody was obtained from Sigma (St. Louis, MO). Alexa Fluor-

488 donkey anti-mouse IgG, Alexa Fluor-555 donkey anti-rabbit IgG and 4',6-diamidino-2-phenylindole-dihydrochloride (DAPI) were purchased from Molecular Probes Inc. (Eugene, OR). RPMI 1640, Dulbecco's phosphate buffered saline (PBS) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA).

3.2 Synovial fibroblast culture

Human RA synovial fibroblasts were isolated from synovial tissue obtained from RA patients who had undergone synovectomy or total joint replacement surgery. The protocol for patient consent and the use of human tissues was approved by the Institutional Review Board at both the University of Michigan and the University of Michigan Health Systems. All tissue was obtained with patient consent. Fresh synovial tissues were minced and digested in a solution of dispase, collagenase, and DNase. The cells were cultured in RPMI 1640 supplemented with 10% FBS in 175-mm tissue culture flasks at 37° C in a humidified atmosphere with 5% CO_2. Upon reaching confluence, the cells were passaged by brief trypsinization.[80] Cells were used at passage 5-9, at which time they were a homogeneous, 85-95% confluent population of fibroblasts. The medium was switched to serum free 12-14 hours before the experiments. The concentration of cytokines, chemokines and signaling inhibitors used in the experiments were based on those used by our lab and others. RA synovial fibroblast cell viability with inhibitors using trypan blue exclusion was >95%.

3.3 Animals

MIF -/- mice were generated as described previously,[81] SV129/J WT mice served as controls. Mice were maintained and bred in a specific pathogen-free facility at the

University of Michigan according to the guidelines for animal research. Animal experiments were in concordance with federal law and were performed after approval by the University Committee in Use and Care of Animals.

3.4 Induction of arthritis

3.4.1 Zymosan-induced arthritis

Zymosan-induced arthritis (ZIA) was induced by intraarticular injection of zymosan (*Saccharomyces cerevisiae*) as follows: zymosan was prepared by dissolving 30 mg of zymosan in 1 ml of sterile PBS. The solution was boiled twice and sonicated. Mice were anesthetized with pentobarbital (60 mg/kg body weight, intraperitoneally) and injected with zymosan (10 µl) into each knee joint.[82] After 24 hours, mice were euthanized and ZIA knees were harvested, one knee was homogenized in PBS containing protease inhibitors (Protease Inhibitor Cocktail, Boehringer Mannheim, Mannheim, Germany), using a Polytron homogenizer (Brinkmann, Westbury, NY), while the other knee was stored in frozen tissue matrix (O.C.T. Compound, Tissue-Tek).

3.4.2 Antigen induced arthritis

Antigen induced arthritis (AgIA) was induced in MIF -/- and WT mice as described previously.[83] The AgIA model involves both cellular and humoral immune responses and shows histological similarities to human RA. Briefly, mice were immunized at day 0 with 200 µg of methylated bovine serum albumin (mBSA, Sigma) emulsified in 0.2 ml of complete Freund's adjuvant injected subcutaneously in the flank skin. At day 7, mice received 100 µg mBSA/0.1 ml Freund's complete adjuvant by intradermal injection at the

base of the tail. At day 21, arthritis was induced by intra-articular injection of mBSA (30 µg in 10 µl of sterile PBS) in the knee. On day 28, mice were euthanized and AgIA knees were harvested and homogenized in PBS containing protease inhibitors.

3.5 Quantitation of MMPs by enzyme immunoassay

MMP-1, MMP-2, MMP-3 and MMP-13 concentrations in cell culture supernatants and the MMP-2 concentration of mouse knee homogenates were measured using Quantikine Immunoassay kits (R&D Systems) according to the manufacturer's instructions. To maintain equal protein loading, protein concentration of ZIA knee homogenates were determined using a bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). The ELISA assays detect both the pro-form and active form of MMP-2, MMP-3, and solely the pro-form of MMP-1 and MMP-13.

3.6 Cell number determination

We used CyQuant cell enumeration kit (Invitrogen, Carlsbad, CA) to monitor equal cell numbers in the presence or absence of stimuli. 10^4 RA synovial fibroblasts were plated into 96-well plates in RPMI containing 10% FBS. The night before the assay, the medium was replaced with serum free RPMI. Cells were incubated in the presence or absence of MIF (50 nM) for 24 hours and cell number was evaluated with CyQuant. Fluorescence background in CyQuant-treated wells without cells was substracted from all values.

3.7 Cell lysis and Western blotting

After treatment with MIF, cells were lysed with lysis buffer (175 µl) (Cell Lysis Buffer, Cell

Signaling Technology, Beverly, MA) containing protease inhibitors. The concentration of protein in each extract was determined using a BCA protein assay using bovine serum albumin as standard. SDS-polyacrylamide gel electrophoresis was performed with cell lysates after equal protein loading, according to the method of Laemmli[84] and proteins were transferred onto a nitrocellulose membrane using a semi-dry transblotting apparatus (Bio-Rad, Hercules, CA). Nitrocellulose membranes were blocked with 5% non-fat milk in Tris-buffered saline Tween buffer (TBST; 20 mM Tris, 137 mM NaCl, pH 7.6, with 0.1% Tween 20) for 60 min at room temperature. Blots were incubated with phospho-specific antibodies at 1:1000 in TBST containing 5% nonfat milk overnight at 4° C. Blots were washed with TBST (3x) for 10 minutes each and incubated with anti-rabbit horseradish peroxidase-conjugated antibodies at room temperature. After washing three times for 10 minutes with TBST, blots were incubated with enhanced chemiluminescence (ECL) reagents (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. The immunoblots were stripped and reprobed with rabbit anti-β-actin to verify equal loading.

3.8 Gelatinase Assay

Gelatinase activity of RA synovial fibroblast culture media was measured using EnzChek Gelatinase Assay Kits (Invitrogen). Cell culture supernatants were incubated with fluorescein conjugated gelatin (100 µg/ml) for 3 hours and fluorescence was measured using a Synergie HT Microplate Reader at 495 nm (Biotek, Winooski, VT).

3.9 Gelatin zymography

MMP-2 enzyme secreted by RA synovial fibroblasts was analyzed on gelatin containing

gels as previously described.[85] . Additionally, gelatin degradation was visualized in AgIA joint homogenates after equalizing protein concentration using BCA protein assay. To the standard acrylamide mixture, B type gelatin (Sigma) was added to a final concentration of 1 mg/ml. Samples were mixed with an equal volume of 2x sample buffer (10% SDS, 10% glycerol, 0.5 M Tris-HCl, pH 6.8 and 0.1% bromophenol blue), then added to 7.5% SDS-polyacrylamide gels, for 2 hours. Following electrophoresis, gels were renatured in 2.5% Triton X-100 for 1 hour at room temperature. The gels were then incubated at 37^{o} C overnight in developing buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$). Gels were stained for 3 hours with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA, USA), then destained with destaining solution (7.5% acetic acid, 5% methanol). Gelatinase activities were visualized as white bands on the blue background of the gels. Molecular weight marker (Sigmamarker, Sigma) and rhMMP-2 was used as a control. Photographs of the zymograms were taken with a Nikon Coolpix 4500 digital camera.

3.10 Immunohistology

Frozen tissue (WT and MIF -/- mouse ZIA joints) were cut (approximately 7 μm) and stained using alkaline-phosphatase and fast red substrate visualization. Slides were fixed in cold acetone for 10 min and then rehydrated with TBS solution for 2 min. Tissues were blocked with 20% FBS, 5% goat serum (in TBS) for 15 min at room temperature, and then incubated with rabbit anti-mouse MMP-2 (diluted 1:200) or purified nonspecific rabbit IgG for 1 hour at room temperature in blocking buffer. The tissue was washed three times in TBS, and a 1:100 dilution (in blocking buffer) of goat anti-rabbit alkaline-phosphatase conjugated antibody was added to the tissue sections and incubated for an additional 1 hour. After washing three times in TBS, slides were developed with Naphtol AS-MX Phosphate and Fast Red TR Salt (20 min at room temperature) (Pierce), rinsed in tap

water, counterstained with Gill's hematoxylin, and dipped in saturated lithium carbonate solution for bluing. Staining was evaluated under blinded conditions and graded by a pathologist. Slides were examined for cellular immunoreactivity and cell types were distinguished based on their characteristic morphology. The percentage of cells expressing MMP-2 was analyzed in the synovial lining cells (fibroblast-like and macrophage-like synoviocytes), on the subsynovial non-lymphoid mononuclear cells (monocytes, macrophages, mast cells) and on endothelial cells.

3.11 Immunofluorescence staining

RA synovial fibroblasts were plated at 3,000 cells/well in 8-well chamber slides in RPMI with 5% FBS overnight. The next day, the media was changed to serum free RPMI. After serum starvation overnight, cells were stimulated with MIF (50 nM) for 20 minutes. Media was aspirated and cells were washed with PBS and fixed with ice-cold methanol for 30 minutes. Blocking was performed by adding 5% FBS in PBS for 1 hour at room temperature. Phospho-specific primary antibodies for JNK and c-jun, or anti-MMP-2 antibody (diluted 1:50), were added overnight at 4° C in blocking buffer. Cells were washed with PBS three times for 5 minutes each. Alexa Fluor-conjugated secondary antibodies, diluted 1:200 in blocking buffer, were added for 1 hour at room temperature. Cells were washed with PBS three times for 5 minutes, then 4',6-diaminido-2-phenylindole (DAPI) nuclear stain was added for 5 minutes in 1:2000 dilution. Slides were dehydrated, mounted and covered with coverslips. Immunofluorescence staining was detected using an Olympus BX51 Fluorescence Microscope System with DP Manager imaging software (Olympus America, Melville, NY).

3.12 MTT viability assay

Effect of EGCG on RA synovial fibroblast viability was determined by MTT viability assay. The results of an MTT-based viability assay using samples obtained from 3 different donors showed that EGCG (10-60 μM) had no significant effect on the viability of cultured RA synovial fibroblasts (data not shown). The highest concentration used in the viability assay (60 μM) showed a mean ± SEM 10 ± 4.0% loss in viability, which was not statistically significant ($p>0.05$).

3.13 Statistical Analysis

Data were analyzed using Student's *t*-test assuming equal variance. *P* values less than 0.05 were considered statistically significant. Data are represented as the mean ± standard error of the mean (SEM).

4. RESULTS

4.1 The effects of MIF on RA synovial fibroblast MMP-2 production

4.1.1 MIF induces the production of MMP-2 in RA synovial fibroblasts

RA synovial fibroblasts were stimulated with MIF (50 nM) for different time periods (6, 24 and 48 hours). Pro-MMP-1, total MMP-2 (pro-form and active form), total MMP-3 and pro-MMP-13 concentrations in cell culture supernatants were measured by ELISA. Under the conditions described above, MIF stimulation showed no effect on MMP-1, MMP-3 and MMP-13 protein secretion as these proteins were not detected in 48-hour MIF-stimulated RA synovial fibroblast culture media by ELISA, whereas control experiments with TNF-α (1.5 nM) increased the concentration of MMP-1, MMP-3 and MMP-13 in the supernatants (data not shown). In contrast, MIF-stimulated RA synovial fibroblasts produced significantly higher amounts of MMP-2 protein compared to non-stimulated controls. This effect was seen after 6 hours stimulation (non-stimulated: 7.13 ± 0.86 ng/ml; MIF stimulated: 16.28 ± 1.71 ng/ml; $p<0.05$) and after 24 hours stimulation as well (non-stimulated: 23.88 ± 7.49 ng/ml; MIF stimulated: 51.36 ± 5.55 ng/ml; $p<0.05$) (Figure 1A).

4.1.2 MIF induces activation of RA synovial fibroblast MMP-2

To analyze enzymatic activity of RA synovial fibroblast supernatants, gelatinase assay was performed using fluorescein labeled gelatin as substrate. Fluorescence intensity was determined in cell culture supernatants of RA synovial fibroblasts stimulated with MIF (50

nM) for 24 hours. Gelatinase assay confirmed enhanced enzymatic activity of MIF-stimulated compared to non-stimulated RA synovial fibroblast supernatants (649 ± 34 mean fluorescence, vs. 503 ± 19; $p<0.05$) (Figure 1B).

Gelatin zymography was performed to visualize the gelatin degradation mediated by MIF in RA synovial fibroblast culture media (Figure 1C). RA synovial fibroblasts were stimulated with TNF-α (1.5 nM)[86] or MIF (50 nM) for 24 hours. Zymography revealed a band of gelatin degradation at 72 kDa, representing pro-MMP-2 protein.

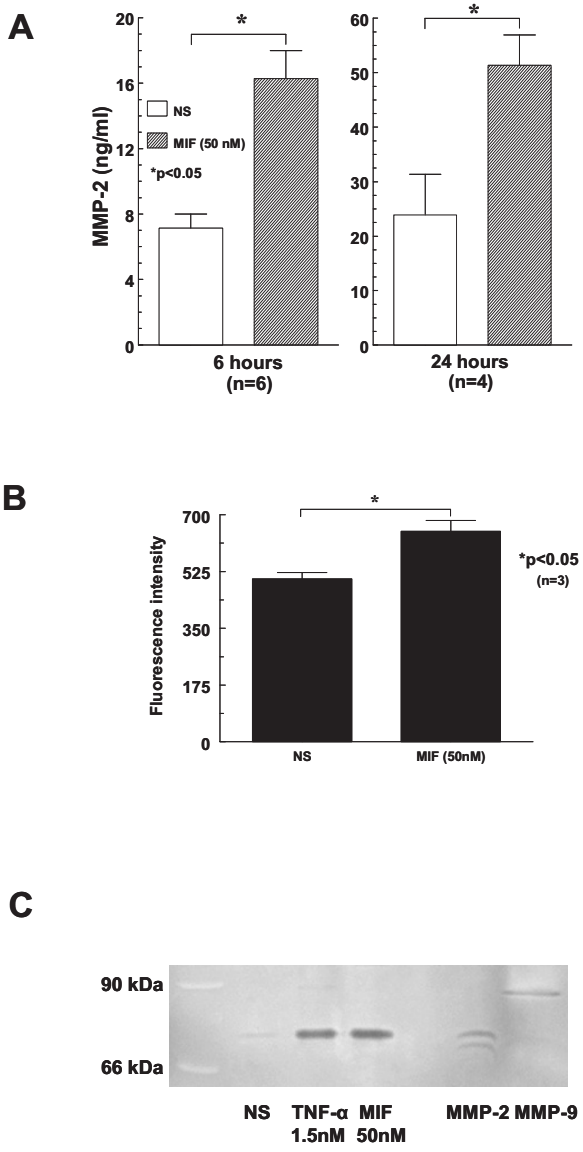

Figure 1. MIF induces MMP-2 production and activity by RA synovial fibroblasts. A. ELISA. B. Gelatinase activity using fluorescein conjugated gelatin as substrate. C. Gelatin zymography. Molecular weight marker and standard recombinant human MMP-2 and MMP-9 (MMP-2, MMP-9) served as control. NS=non-stimulated, n=number of donors

4.1.3 MIF-induced MMP-2 production is time- and concentration dependent

We stimulated RA synovial fibroblasts with MIF (50 nM) for different time periods (1, 3, 6, 12 and 24 hours). MMP-2 secretion was visualized in supernatants by zymography (Figure 2A). We found that MIF-induced RA synovial fibroblast MMP-2 upregulation was time dependent beginning at 1 hour and increasing continuously through 24 hours.

Using immunofluorescence staining, we showed intracellular MMP-2 upregulation after 1 hour stimulation by MIF (Figure 2B), confirming the role of MIF in MMP-2 induction. Immunofluorescence staining for MMP-2 showed a strong perinuclear and discrete diffuse cytoplasmic pattern.

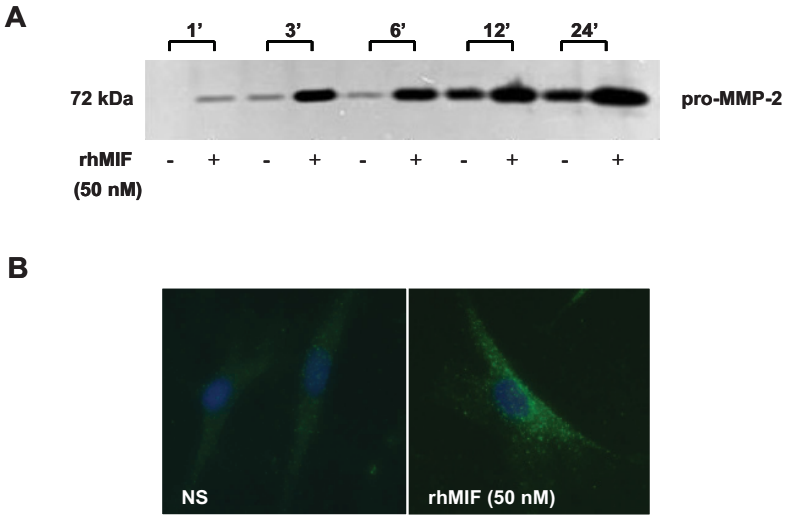

Figure 2. MMP-2 upregulation by MIF is time dependent. A. Gelatin zymography B. Immunofluorescence staining of RA synovial fibroblasts for MMP-2 (400X). NS=non-stimulated

After RA synovial fibroblasts were stimulated with different concentrations of MIF (1, 5, 10, 25 and 50 nM), MMP-2 content of the cell culture supernatants was determined by gelatin zymography. We observed no stimulatory effect at 1 nM MIF, while increasing MMP-2 expression was seen in response to higher concentration of MIF. Similarly elevated MMP-2 levels were observed at concentrations of 25 and 50 nM MIF.

4.2 Signaling pathways involved in MIF-induced RA synovial fibroblast MMP-2 production

4.2.1 Signaling molecules involved in MIF-induced MMP-2 expression

To examine the signal transduction pathways induced by MIF, RA synovial fibroblasts were stimulated with MIF (50 nM) in the presence of different signaling inhibitors. MMP-2 concentrations in RA synovial fibroblast supernatants were measured by ELISA (Figure 3A) and gelatin degradation was visualized by gelatin zymography (Figure 3B). Several inhibitors were tested, including the PKC inhibitor Ro31-84-25 (1 μM), the PKA inhibitor H-8 (10 μM), the MEK inhibitor PD98059 (10 μM), the p38 MAPK inhibitor SB203580 (10 μM), the PI3K inhibitor LY29002 (10 μM), the Src inhibitor PP2 (10 μM), the JAK inhibitor AG 490 (10 μM), the NFκB inhibitor PDTC (100 μM), the JNK inhibitor SP600125 (10 μM), the G-protein inhibitor pertussis toxin (4.3 nM) and the STAT inhibitor peptide (100 μM). We observed that MMP-2 upregulation by MIF was suppressed by inhibitors against pan-PKC, JNK and in part by Src, suggesting that these signaling pathways are involved in MMP-2 production by RA synovial fibroblasts. In contrast, none of the other specific signaling inhibitors mentioned above reduced MMP-2 expression of MIF-stimulated RA synovial fibroblasts.

In order to determine the role of different PKC isoforms in MMP-2 production we used a PKCα/β specific inhibitor Gö6976 (1 μM) and a PKCδ isoform specific inhibitor rottlerin (1 μM). We found that rottlerin inhibited the upregulation of MMP-2 by MIF, while the α and β isoform-specific PKC inhibitor Gö6976 did not.

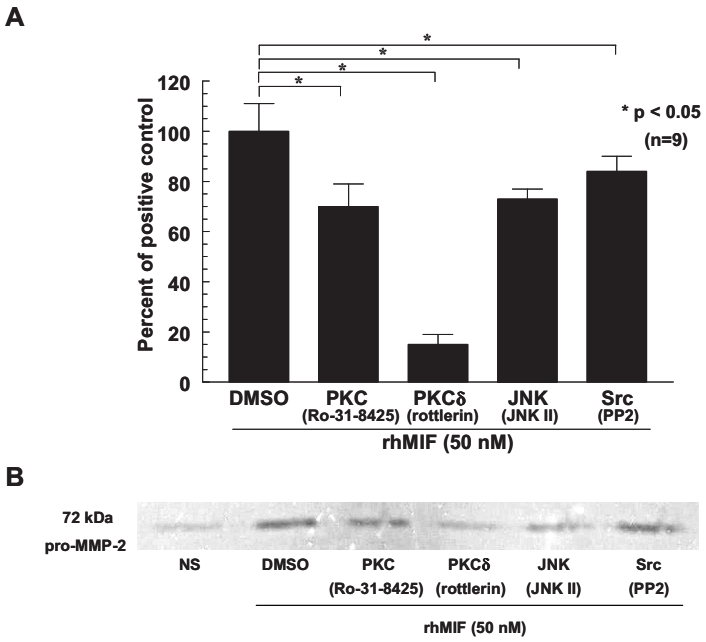

Figure 3. Signaling pathways involved in MIF-induced RA synovial fibroblast MMP-2 production. A. ELISA B. Gelatin zymography. NS=non-stimulated, n=number of donors

4.2.2 Signaling molecules activated by MIF

In order to study signal transduction, we used MIF at a concentration of 25 nM, as we determined this dose to be sufficient for inducing MMP-2 production in RA synovial fibroblasts. RA synovial fibroblasts were stimulated with MIF for different time periods (0, 1, 5, 15, 30 and 45 minutes) (Figure 4). The phosphorylation of JNK and the JNK

substrate c-jun was determined by Western blot using phospho-specific antibodies. MIF activated JNK phosphorylation at 1 minute and a maximum response was seen at 15 minutes (Figure 4A). MIF-induced c-jun activation was observed after 30 minutes (Figure 4B). In order to confirm these data, we performed immunofluorescence staining of RA synovial fibroblasts using antibodies to phospho-specific signaling molecules. We found diffuse cytoplasmic staining of phospho-JNK in RA synovial fibroblasts stimulated by MIF (Figure 4C). The intracellular localization of phospho-c-jun was primarily nuclear but there was also a small amount of cytoplasmic staining. Upon MIF stimulation, immunofluorescence staining of phospho-c-jun showed a stronger nuclear pattern, suggesting nuclear translocation of c-jun (Figure 4D).

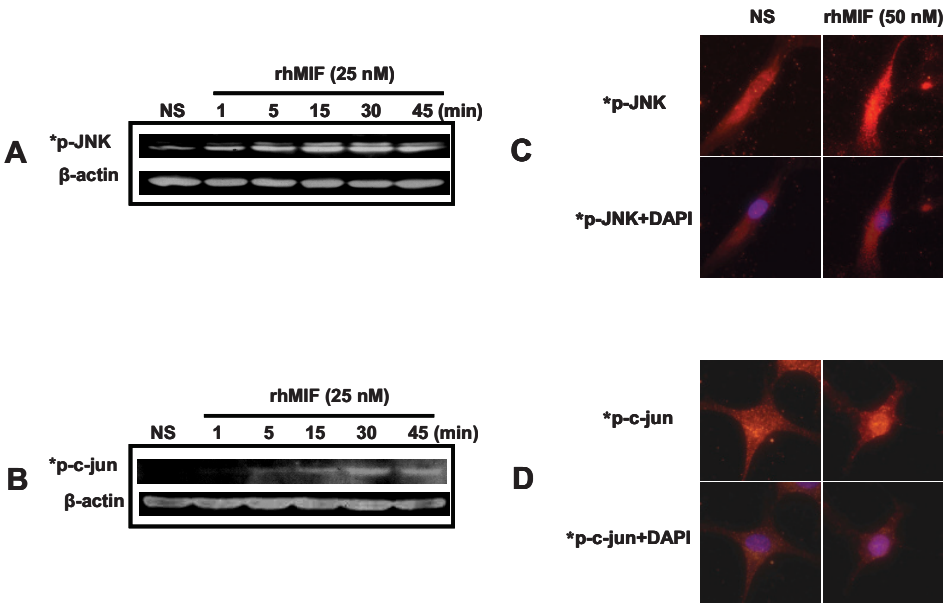

Figure 4. MIF activates JNK and c-jun in RA synovial fibroblasts. A-B. Western blot. C-D. Immunofluorescence staining of RA synovial fibroblasts shows the expression of different phospho-signaling molecules in the presence or absence of MIF (50 nM) for 25 minutes and with or without DAPI nuclear staining. (400X) NS=non-stimulated, *p=phospho

In order to determine which PKC isoforms are phosphorylated upon MIF stimulation we used different anti-phospho-specific PKC antibodies (Figure 5). We did not find activation of pan-PKC, PKCα_I/β_{II} and PKCε isoforms upon MIF stimulation (Figure 5A-C). Also, MIF did not induce phosphorylation of PKCδ at tyrosine 311 (Figure 5D), but specifically induced phosphorylation of PKCδ at threonine 505 (Figure 5E). The reason for this effect may be that PKCδ is not phosphorylated at Tyr 311, but rather at Thr 505. MIF-induced activation of this PKC isoform was found after 1 minute with a maximum response between 30-45 minutes.

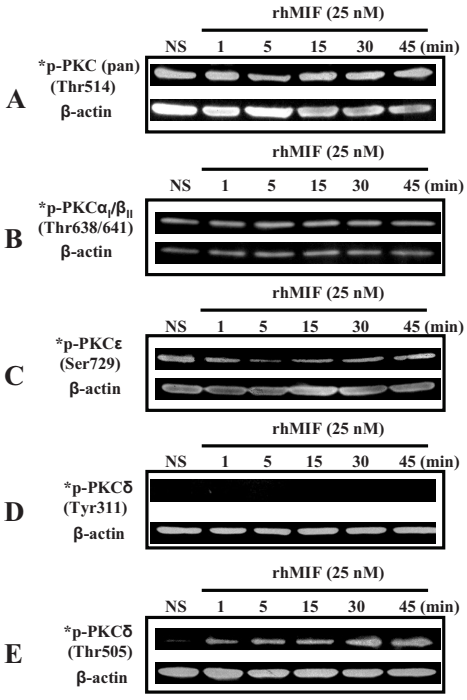

Figure 5. MIF activates PKCδ. Western blots were performed using phospho-specific antibodies against A. *P-PKC (pan), B. *p-PKCα_I/β_{II} and C. *p-PKCε. MIF induced the phosphorylation of PKCδ at threonine 505 (E), but not on tyrosine 311 (D).

4.2.3 The signaling cascade

We wished to determine the downstream and upstream signaling mechanisms, therefore RA synovial fibroblasts were incubated with signaling inhibitors 1 hour prior to MIF stimulation (25 nM). Phosphorylation of JNK was abrogated by the Src inhibitor PP2 (Figure 6A), and c-jun phosphorylation was abrogated by JNK and Src inhibitors (Figure 6B). These data suggest that Src is upstream of JNK, and phosphorylation of JNK leads to the activation of the nuclear protein c-jun. Activation of PKCδ (Thr505) was inhibited by JNK and Src inhibitors (Figure 6C), suggesting that Src and JNK are upstream of PKCδ. The inhibitory activity of rottlerin is due to the interaction with the ATP-binding site of PKCδ, which explains why it did not inhibit the phosphorylation of PKCδ [87]. (Figure 7)

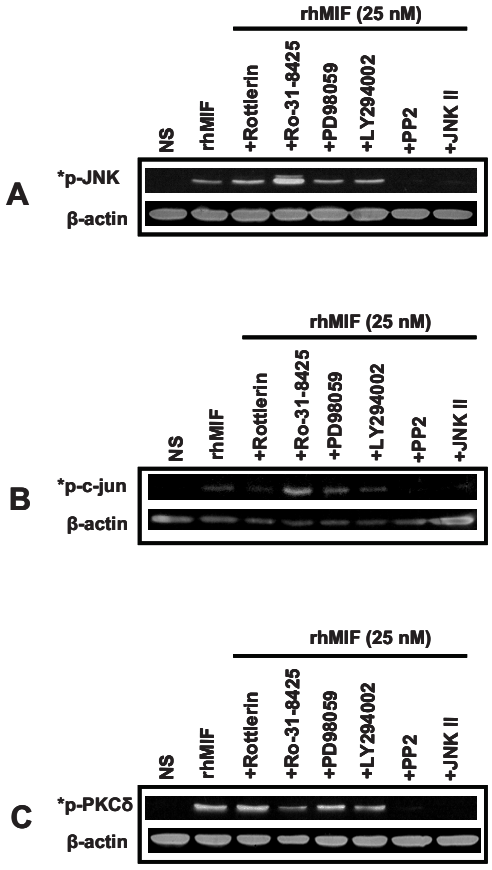

Figure 6. Signaling cascade activated by MIF. **A.** Upregulation of *p-JNK by MIF was inhibited by a Src inhibitor PP2. **B.** The activation of the nuclear factor c-jun required the phosphorylation of JNK and Src. **C.** Similarly, *p-PKCδ expression was Src and JNK pathway dependent. NS=non-stimulated, *p=phospho

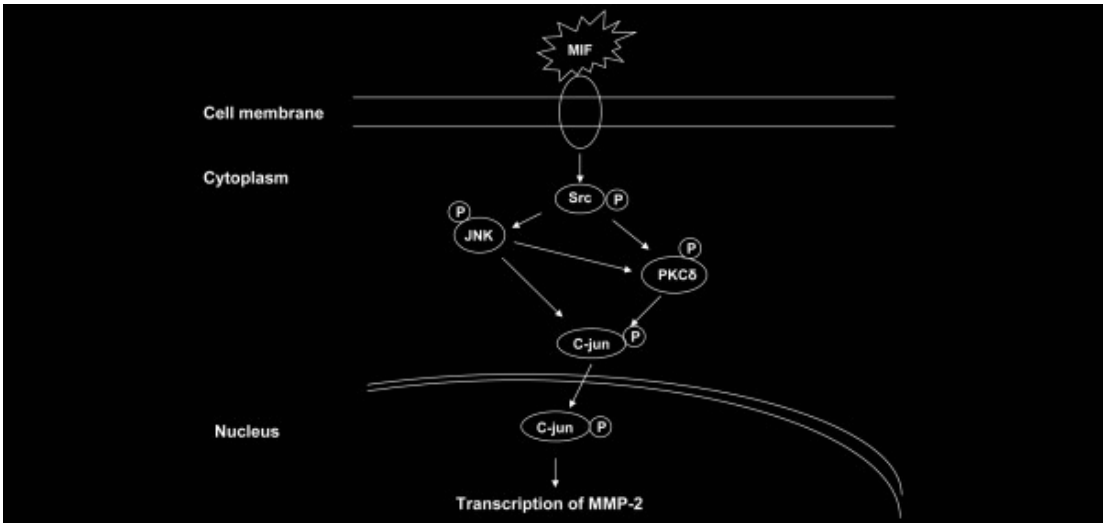

Figure 7. A schematic model of signaling pathways involved in MIF-induced MMP-2 expression in rheumatoid arthritis (RA) synovial fibroblasts

4.3 The role of MIF in *in vivo* MMP-2 production

4.3.1 MMP-2 production in acute inflammatory arthritis

In order to evaluate the *in vivo* role of MIF in MMP-2 production, we induced acute arthritis by intraarticular injection of zymosan in MIF -/- and WT mice. ZIA is a model of acute inflammatory arthritis with early (day 1) and late phases.[88] After 24 hours, mice were euthanized; ZIA knee joints were harvested and homogenized. To compare MMP-2 production in joints of MIF -/- and WT mice, MMP-2 concentrations of knee homogenates were measured by ELISA and normalized to total protein. We found significantly elevated MMP-2 protein levels in knee homogenates of WT mice compared to MIF -/- mice (WT: 1.3 ± 0.08 ng/mg protein; MIF -/-: 0.82 ± 0.08 ng/mg; mean ± SEM, $p<0.05$), pointing to an important role of MMP-2 in arthritis (Figure 8A).

4.3.2 MMP-2 expression in AgIA

In addition, we measured MMP-2 expression in the knee joints of mice after induction of AgIA, a different murine model of RA. MMP-2 production of knee homogenates was measured on day 28, after AgIA induction using gelatin zymography (Figure 8B).

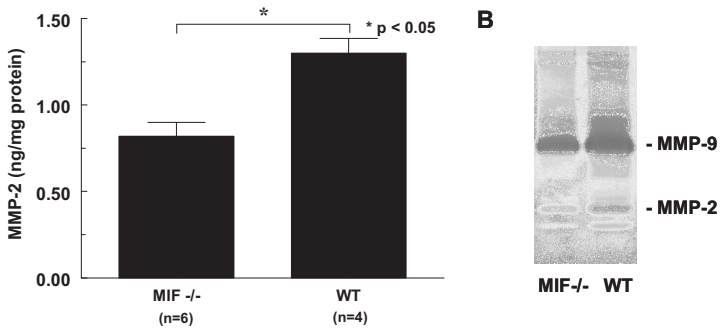

Figure 8. MMP-2 production is decreased in MIF -/- mice. A. In ZIA measured by ELISA. B. In AgIA using gelatin zymography. n=number of mice per group

In concordance with our previous results, zymography showed enhanced MMP-2 expression (both pro- and active forms of MMP-2) in WT mice compared to MIF -/- ones. Interestingly, zymography revealed both pro- and active forms of MMP-2.

4.3.3 Immunohistological analysis of MMP-2 expression in inflamed joints

In order to evaluate the cell-type specific expression of MMP-2 in the synovium of arthritic joints, we performed immunohistochemistry staining on ZIA joints of MIF -/- and WT mice. ZIA was induced as we described above, knee joints were kept in OCT and frozen joint sections were immunoassayed for MMP-2. We found that MMP-2 was mainly expressed

by synovial lining cells (Figure 9 A-C), sublining non-lymphoid mononuclear cells and by endothelial cells. The immunostaining was quantified as the percentage of cells staining positively for MMP-2. Synovial expression of MMP-2 was enhanced on both lining cells (Figure 9D) and sublining non-lymphoid mononuclear cells (Figure 9E) of WT compared to MIF -/- mice (synovial lining cells: 74% ± 7 vs. 38% ± 7, sublining non-lymphoid mononuclear cells: 72% ± 4.9 vs. 22% ± 3.8; mean ± SEM, $p<0.05$). A similar trend was seen in case of endothelial cells, however the difference was not significant (41% ± 13.5 vs. 14.4% ± 4.8; mean ± SEM) (Figure 9F).

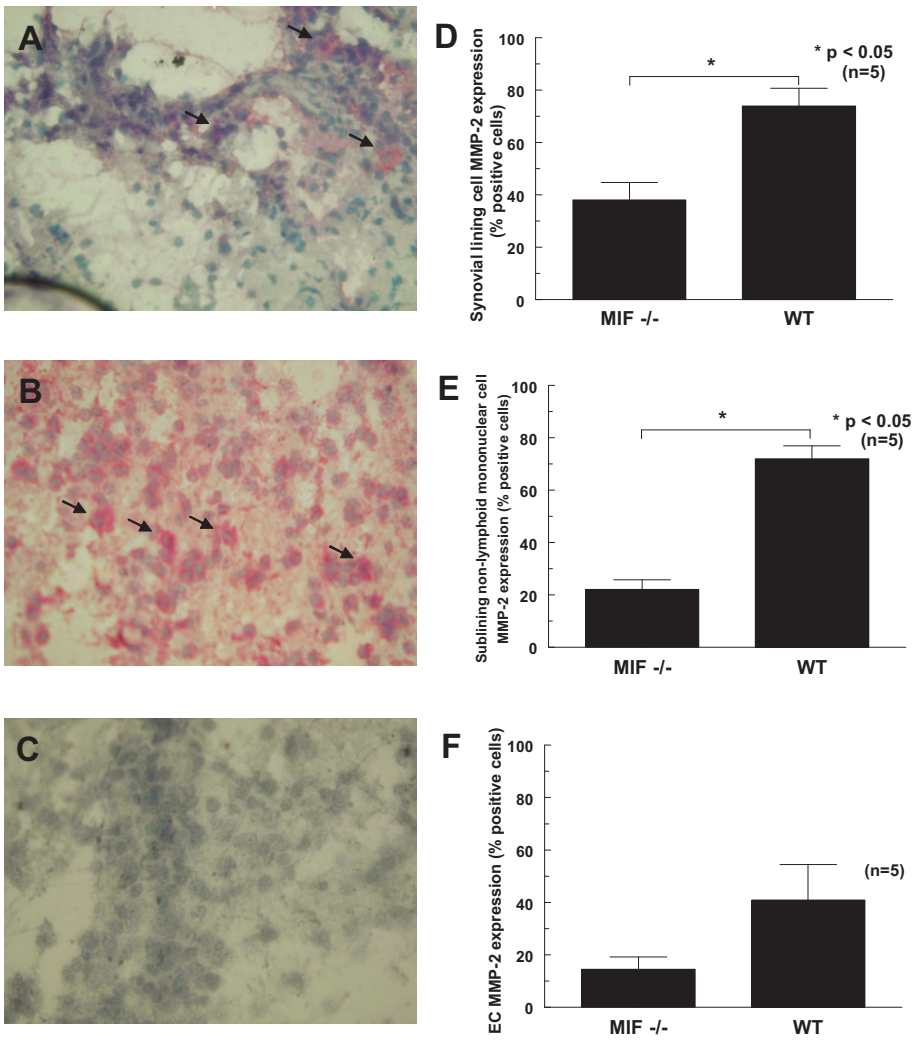

Figure 9. MMP-2 expression by synovial lining cells in ZIA joints. MMP-2 expression was decreased in synovial lining cells, which are composed of macrophages and fibroblasts, of MIF -/- (A) compared to WT (B) mice. Irrelevant IgG was used as the negative control (C) (400x). Black arrows indicate synovial lining cells stained for MMP-2. D-F. The average percentage of cells stained for MMP-2. D. Synovial lining cells E. Sublining non-lymphoid mononuclear cells. F. Endothelial cells (EC). n=number of animals in each group

4.4 Effect of EGCG on RA synovial fibroblast MMP-2 production

In order to study the effect of EGCG on MMP-2 production, RA synovial fibroblasts were incubated with or without EGCG (10-20 μM) in serum-free medium for 12 hours, followed by stimulation with IL-1β (10 ng/ml) for 24 hours. After 24 hours, culture supernatant was collected and centrifuged at 10,000*g* for 5 minutes at 4℃ to remove particulate matter, and stored at -80℃ in fresh tubes. MMP-2 activity in conditioned medium was measured using gelatin zymography. Stimulation of RA synovial fibroblasts with IL-1β resulted in a 2.5-fold increase in MMP-2 activity ($p<0.05$) (Figure 10). Treatment of RA synovial fibroblasts with EGCG at concentrations of 10 μM and 20 μM resulted in a mean ± SEM 58 ± 14% and 75 ± 16% inhibition of IL-1β-induced MMP-2 activity, respectively ($p<0.05$). Interestingly, the fibroblasts treated with EGCG (10 μM and 20 μM) alone showed a marked inhibition of MMP-2 activity (76 ± 22% and 70 ± 20%, respectively), when compared with the activity observed in untreated samples ($p<0.05$).

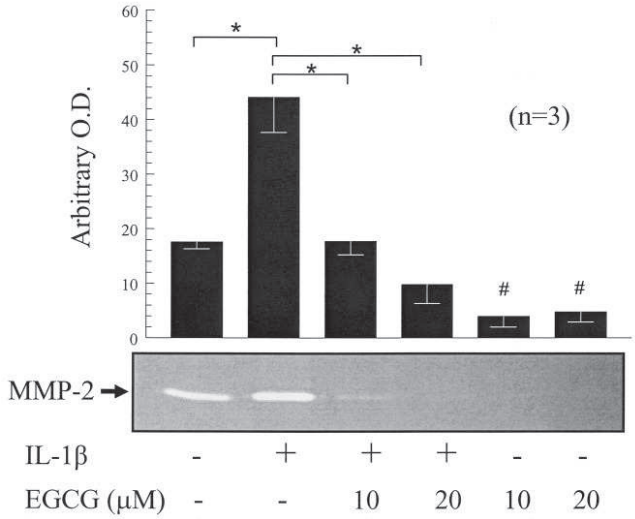

Figure 10. Inhibition of constitutive and IL-1β-induced MMP-2 activity by EGCG in RA synovial fibroblasts. MMP-2 activity in the cell-free supernatants was determined by gelatin zymography. * $p<0.05$ vs. treatment with IL-1β alone; # $p<0.05$ vs. untreated control. OD = optical density

4.5 Effect of chemokines and EGCG on RA synovial fibroblast MMP-2 activity

4.5.1 Effects of chemokines on RA synovial fibroblast MMP-2 expression

RA synovial fibroblasts were stimulated with ENA-78/CXCL5 (100 ng/ml), gro-α/CXCL1 (100 ng/ml), or RANTES/CCL5 (100 ng/ml) for 24 hours. We found that ENA-78/CXCL5, gro-α/CXCL1, and RANTES/CCL5 increased RA synovial fibroblast MMP-2 activity by 3.9-, 5.4-, and 4.9-fold, respectively, as compared with untreated controls ($p<0.05$) (Figure 11). MMP-2 activity induced by these three chemokines was 1.1-, 1.5-, and 1.3-fold higher, respectively, in comparison to IL-1β-stimulated samples.

4.5.2 Effects of EGCG on chemokine-induced RA synovial fibroblast MMP-2 expression

Treatment of RA synovial fibroblasts with EGCG markedly inhibited the ability of chemokines to stimulate MMP-2 activity, in a concentration-dependent manner. EGCG at 10 μM and 20 μM blocked MMP-2 activity induced by ENA-78 by 33% and 75%, by gro-α by 52% and 80%, and by RANTES by 55% and 84%, respectively ($p<0.05$) (Figure 11).

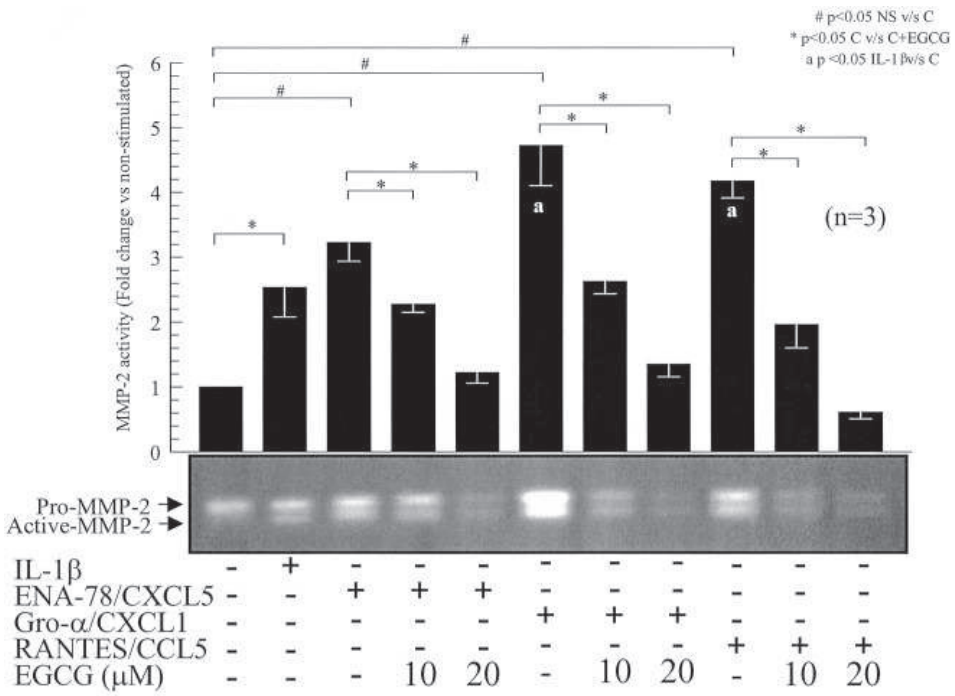

Figure 11. Inhibition of chemokine-induced MMP-2 activity by EGCG in RA synovial fibroblasts**.** MMP-2 activity in the cell-free supernatants was determined by gelatin zymography. NS = not stimulated; C = chemokines

5. DISCUSSION

Functional disability of the joints as a consequence of progressive cartilage and bone degradation is a hallmark of RA. Known effector molecules in the destruction of articular cartilage and bone are MMPs and cathepsins. These enzymes have been implicated in several pathological processes such as tumor invasion, angiogenesis, atherosclerosis, and RA.[4,22,23,25-28]. In RA, angiogenesis is considered as a key event in the expansion of the synovial lining of the joints. Angiogenesis requires proteolysis of the extracellular matrix, proliferation, and migration of endothelial cells, as well as synthesis of new matrix components.[8,11]

RA synovial fibroblasts at sites of invasion or within the synovial lining layer are a major source of MMPs, and drive RA joint destruction as well as angiogenesis via these enzymes. MMPs are a group of Zn^{2+} dependent extracellular enzymes that have a key role in a normal and pathological tissue remodelling. The whole group can be divided into subclasses, such as collagenases, gelatinases, stromelysins, and membrane type MMPs. MMPs have the ability to degrade extracellular matrix components including gelatin, collagens, fibronectin, and laminin.[29]

Although the function of MMP-2 in RA has not yet been fully elucidated, several studies showed an important role of MMP-2 in RA. Increased levels of MMP-2 were detected in sera and synovial fluids of patients with RA,[34] and increased MMP-2 production has been associated with enhanced RA synovial fibroblast invasion.[33] In addition, MMP-2 also participates in angiogenesis.[4-6] On the other hand, gene polymorphisms of MMP-2 may affect susceptibility to development and/or severity of RA, and mutation of the MMP-2

gene causes a multicentric osteolysis and arthritis syndrome.[89] Thus, in the present study, we wished to better understand the cellular and molecular basis of MMP-2 production by RA synovial fibroblasts *in vitro* and *in vivo*. Further, we also searched for a potent inhibitor of RA synovial fibroblast MMP-2 expression.

MIF is highly expressed in RA synovium[90] and regulates the production of proinflammatory cytokines, such as TNF-α, IL-1β and IFN-γ[39]. MIF also induces the release of MMP-1 and MMP-3 by RA synovial fibroblasts.[91] Thus, MIF is thought to be important in the pathogenesis of RA. The angiogenic potential of MIF was previously demonstrated both *in vitro* and *in vivo*. MIF induces human dermal microvascular endothelial cell migration and tube formation, and induces angiogenesis in Matrigel plugs, as well as in the corneal bioassay *in vivo.*[44] Two groups observed independently that in MIF -/- or in WT mice treated with neutralizing antibody against MIF, the onset of arthritis was delayed and synovial inflammation was decreased[45,48], although the specific role of MIF in tissue destruction has not yet been clarified. In the present study, a novel role of MIF, its ability to induce MMP-2 production by RA synovial fibroblasts and MIF-induced signaling events were analyzed.

First, we investigated the effect of MIF on RA synovial fibroblast MMP production. Previously, Onodera et al[91,92] showed a stimulatory effect of MIF on MMP-1 and MMP-3 mRNA levels in RA synovial fibroblasts, and also on MMP-9 and MMP-13 production in rat osteoblasts. In contrast to Onodera et al[91], who found increased levels of MMP-1 protein in supernatants of MIF-stimulated early passage RA synovial fibroblasts, we found no upregulation of MMP-1, MMP-3 and MMP-13 production by MIF by later passage cells. It is possible that these differences in responsiveness may be due to differences in cell passage number, as similar *in vitro* aging effects were shown previously.[93]

Cell invasion and angiogenesis are crucial processes underlying diseases such as RA and cancer. Previously, Meyer-Siegler et al[94] reported a positive correlation between MIF and MMP-2 expression in prostate cancer cells. MIF addition to proliferating DU-145 prostate cancer cells resulted in a two-fold increase in the relative amount of active MMP-2. In this study, we showed that MIF induced MMP-2 production by RA synovial fibroblasts, which may lead to joint destruction in RA. Using *in vitro* methods, including gelatin zymography, ELISA, and immunofluorescence staining of RA synovial fibroblasts, we observed that MIF induced MMP-2 production by RA synovial fibroblasts. Stimulation of RA synovial fibroblasts by MIF resulted in a two-fold increase in MMP-2 production. In addition, MIF enhanced the gelatinase activity of RA synovial fibroblast-secreted proteins. Zymography analysis demonstrated the increase of pro-MMP-2 protein level by RA synovial fibroblasts stimulated by MIF. It is known, that fibroblasts alone routinely release MMP-2 in a pro-form. However, co-culture of fibroblasts and monocytes results in the activation of pro-MMP-2[32,95]. Among other factors, neutrophil elastase is also known to augment the conversion of 72 kDa MMP-2 to the 66 kDa form in lung fibroblasts[95,96].

In order to evaluate the *in vivo* role of MIF in MMP-2 production, we induced acute inflammatory arthritis in MIF -/- and WT mice by zymosan. The synovial inflammation mediated by zymosan is biphasic, with an initial peak at day 1, followed by a continuous decrease and a secondary increase at day 14, as previously described using quantification of joint inflammation *in vivo.*[88] Our results confirmed the important role of MIF in MMP-2 production, as MIF -/- mice expressed less joint MMP-2 than WT mice. This observation may contribute to a less severe arthritis in MIF -/- mice compared to WT mice described previously.[45,48,97] In parallel with these results, we measured MMP-2 levels in AgIA, a murine model of RA, using gelatin zymography. We found that both the pro- and

active forms of MMP-2 were present in the joints and both forms were upregulated in WT compared to MIF -/- mice. Along with previous monocyte and fibroblast co-culture studies,[32,95,96] our results also suggest that activation of MMP-2 produced by RA synovial fibroblasts requires the presence of other cell types, possibly monocytes or neutrophils.

Immunohistochemical analysis of ZIA joints revealed that MMP-2 was mainly expressed by synovial lining cells, non-lymphoid mononuclear cells and endothelial cells in the synovium. In addition, we showed that MMP-2 expression by lining cells and non-lymphoid mononuclear cells was upregulated in WT compared to MIF -/- animals, suggesting an important role of MIF in MMP-2 induction by these cells. Regarding *in vivo* studies, it was previously shown that progressive joint destruction could be prevented by a novel synthetic MMP inhibitor in rat adjuvant induced arthritis and also in collagen-induced arthritis.[98,99] In contrast, in the antibody-induced arthritis model, synovial inflammation was found to be more severe in MMP-2 -/- mice compared to WT animals.[100]

We also assessed specific signaling pathways mediating MIF-induced MMP-2 production in RA synovial fibroblasts. We found that MIF-induced RA synovial fibroblast MMP-2 production was decreased in the presence of inhibitors of JNK, PKC and Src signaling pathways. Pretreatment of RA synovial fibroblasts with a PKCδ isoform specific inhibitor rottlerin suppressed MIF-induced MMP-2 upregulation. Interestingly, we also found that MIF induced the phosphorylation of JNK, c-jun and PKCδ in RA synovial fibroblasts in a time-dependent manner. We also found that activation of JNK and PKCδ by MIF required the interaction of Src. JNK and Src are upstream activators of PKCδ and phosphorylation of JNK leads to the activation of c-jun.

A number of molecules have been implicated in MIF-mediated signaling. Tyrosine

kinases, PKC and NFκB signaling molecules are activated by MIF leading to IL-8 and IL-1β upregulation in RA synovial fibroblasts.[40] MIF-induced MMP-1, MMP-3 and IL-1β mRNA upregulation in RA synovial fibroblasts was inhibited by staurosporine, a broad spectrum inhibitor of protein kinases such as PKC; a tyrosine kinase inhibitor, genistein; a PKC inhibitor, H-7; and a transcription factor AP-1 inhibitor, curcumin.[91] In another study, two tyrosine kinase inhibitors, genistein and herbimycin A, a selective MAPK kinase inhibitor (PD98059), and curcumin inhibited MIF-induced MMP-13 mRNA upregulation in rat osteoblasts.[92] In consistence with these results, in rat osteoblasts MIF stimulates phosphorylation of tyrosine, autophosphorylation of Src, activation of Ras, activation of extracellular ERK1/2, a MAPK, but not c-Jun N-terminal kinase or p38, and phosphorylation of c-Jun.

Other regulatory mechanisms, such as transcriptional and post-transcriptional control of MMP-2 mRNA levels by MIF may also be important, and are currently under investigaton. The transcription factors Sp1, Sp3 and AP-2 are functionally important in regulating the expression of the MMP-2 gene.[101,102] Both Sp1 and AP-2 transcription factors have been implicated in tumor progression,[103,104] as well as angiogenesis.[105] Among these transcription factors, c-jun interacts with Sp1 and the expression of Sp1 is downregulated by the PKCδ inhibitor rottlerin, suggesting a possible interaction of Sp1 with PKCδ.[106]

MMP-2 is involved in RA pathogenesis by assisting RA synovial fibroblasts in the invasion of microvascular basement membrane and the interstitium.[37,38] Chemokine-activated RA synovial fibroblasts may mediate this process by their attachment to the cartilage surface and the release of MMPs.[7] Interestingly, recent findings suggest an active involvement of selective chemokines in the destructive phase of RA, in which migration, proliferation, and

MMP production by RA synovial fibroblasts are characteristic features.[7] Therefore, in the present study, we wished to further characterize the role of chemokines in RA synovial fibroblast MMP-2 production. We showed that certain chemokines such as ENA-78/CXCL5, gro-α/CXCL1 and RANTES/CCL5 are potent inducers of RA synovial fibroblast MMP-2 production. Treatment of RA synovial fibroblast by any of these three chemokines resulted in a 3.9- to 5.4-fold increase in MMP-2 activity of these cells in comparison to untreated control cells.

Targeting chemokines and/or chemokine receptors appears to be an intriguing new approach to treating chronic inflammatory disorders like RA, inflammatory bowel diseases, multiple sclerosis, and transplant rejection. The involvement of chemokines and chemokine receptors in inflammatory joint diseases, the *in vitro* and *in vivo* characteristics of the chemokine family in synovitis, and preliminary clinical data on chemokine blockade in patients with RA suggest that targeting the chemokine and chemokines receptor family might provide a new, promising antirheumatic strategy (Szekanecz Z, et al. Chemokines and angiogenesis in rheumatoid arthritis. Front Biosci, in press).[56,57]

Green tea is rich in catechins, and several epidemiological and animal model studies have shown that green tea consumption was associated with some health benefits including inhibition of inflammation.[107] Most of the beneficial health effects of green tea are mimicked by its most prevalent catechin, EGCG at micromolar concentrations. EGCG influence a number of cellular mechanisms and has been shown to inhibit the activities of MMP-2 and MMP-9 in tumor cells.[108,109] In addition, EGCG is also an inhibitor of metallo-elastase and serine-elastase activity and down-regulates the levels of several markers of oxidative stress.[110,111] In our study, ECGC inhibited IL-1β- and chemokine-induced RA synovial fibroblast MMP-2 expression. Two different doses of EGCG inhibited MMP-2

activity induced by three chemokines by 33-84%.

6. SUMMARY

In summary, we demonstrated an important role for MIF in RA synovial fibroblast MMP-2 production, which may contribute to tissue destruction in RA. *In vivo* MMP-2 upregulation by MIF was investigated in ZIA, an acute inflammatory arthritis model and in AgIA, a murine model for RA, using MIF -/- and WT mice. In addition, we described important pathways involved in MIF-dependent upregulation of MMP-2 production and activity. In our study, we showed that JNK, Src and PKCδ, a novel signaling intermediate, mediated MIF-induced RA synovial fibroblast MMP-2 expression. Furthermore, chemokines including ENA-78/CXCL5, gro-α/CXCL1 and RANTES/CCL5 all induced RA synovial fibroblast MMP-2 expression. These effects could be inhibited by EGCG, a constituent of green tea, suggesting that natural products, as well as pharmacological agents may interfere with inflammatory and destructive processes associated with arthritis.

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8. ABSTRACT

RA is a chronic inflammatory disease characterized by synovial hyperplasia, invasion and destruction of bone and cartilage. In RA, angiogenesis is thought to be a key event in the expansion of the synovial lining of the joints. Angiogenesis requires proteolysis of the extracellular matrix, proliferation, and migration of endothelial cells, as well as synthesis of new matrix components. MMP-2 plays an important role in this angiogenic process.

In the present study, we investigated RA synovial fibroblast MMP-2 production in response to various stimuli. We found that MIF induced RA synovial fibroblast MMP-2 expression in a time and concentration dependent manner. MIF-induced RA synovial fibroblast MMP-2 upregulation required PKCδ, JNK and Src signaling pathways. Consistent with these results, MIF induced phosphorylation of JNK, PKCδ and c-jun. Further, we found that MMP-2 protein levels were significantly decreased in MIF gene deficient mice compared to wild type mice joint homogenates in zymosan induced arthritis. Immunohistochemistry staining revealed that synovial lining cells, endothelial cells and sublining non-lymphoid mononuclear cells expressed MMP-2 in the synovium. Consistent with these results, in antigen-induced arthritis, a model for RA, enhanced joint MMP-2 expression was also observed in wild type compared to MIF gene deficient mice. Furthermore, chemokines including ENA-78/CXCL5, gro-α/CXCL1 and RANTES/CCL5 all induced RA synovial fibroblast MMP-2 expression. These stimulatory effects were inhibited by EGCG, a constituent of green tea, suggesting that natural products, as well as pharmacological agents may interfere with inflammatory and destructive processes associated with arthritis.

Keywords: rheumatoid arthritis, synovial fibroblasts, matrix metalloproteinases, macrophage migration inhibitory factor, chemokines, epigallocatechin-3-gallate

A RA egy krónikus gyulladásos betegség, melyet szinoviális hiperplázia, csont- és porcdestrukció jellemez. A RA patogenezisében kulcsszerepet játszó angiogenezis elősegíti a gyulladásos szinóvium növekedését. Az angiogenezishez elengedhetetlenül szükséges az extracelluláris mátrix proteolízise, proliferációja, az endothel sejtek ízületi szinóviumba történő migrációja és az új mátrix fehérjék szintézise. A MMP-2 kiemelkedő szerepet játszik ebben a folyamatban.

Tanulmányunkban megvizsgáltuk a RA szinoviális fibroblasztok MMP-2 expresszióját különböző stimulusok hatására. Azt találtuk, hogy a MIF stimulálta RA szinoviális fibroblasztok MMP-2 termelését idő és koncentráció függő módon. A MIF által kiváltott RA szinoviális fibroblaszt MMP-2 expresszió PKCδ, JNK és Src jelátviteli útvonalakat igényelt. Ezen eredményekhez hasonlóan, a MIF indukálta a JNK, PKCδ és c-jun foszforilációját. Mindezen túl, a MMP-2 szignifikánsan alacsonyabb szintjét figyeltük meg a MIF gén deficiens egerek ízületi homogenizátumában vad típusú egerekhez képest zymosan-indukálta arthritisben. Az immunhisztokémiai vizsgálatok azt mutatták, hogy a szinoviális felszíni réteg sejtei, a felszín alatti nem limfoid típusú mononukleáris sejtek és az endothel sejtek expresszálnak MMP-2-t a szinóviumban. Hasonló eredményeket találtunk az antigen-indukálta arthritises modellben. A MIF gén deficiens egerekkel összehasonlítva, emelkedett ízületi MMP-2 expressziót figyeltünk meg vad tipusú egerekben. Mindezeken túl, az ENA-78/CXCL5, a gro-α/CXCL1 és a RANTES/CCL5 kemokinek indukálták a RA szinoviális fibroblasztok MMP-2 expresszióját. EGCG, a zöld tea aktív komponense, gátolta a kemokinek által kiváltott hatásokat, amely azt engedi feltételezni, hogy természetes anyagok, ugyanúgy mint egyes farmakológiai hatóanyagok jelentősen gátolhatják a RA-ben lejátszódó gyulladásos és destruktív folyamatokat.

9. JOURNAL PUBLICATIONS

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