Chapter 2

Matrix metalloproteinases in rheumatoid arthritis

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Submitted.
INTRODUCTION

Rheumatoid arthritis (RA) is the most common polyarthritis affecting about 0.5-1% of the general population. Besides a chronic symmetrical polyarthritis of small joints in hands and feet, larger joints are generally involved and extra-articular manifestations are frequently present. It is a complex inflammatory auto-immune disease, associated with considerable disability, morbidity, and mortality.

I. Pathogenesis of RA

Despite intensive research the exact cause of RA remains a mystery. A mixture of environmental and genetic factors seems to be of importance. Environmental pathogens may infect an individual with a predisposing genetic background and by an unknown mechanism the subsequent inflammatory response is mainly targeted to the synovial joints.

Based on data from animal models it is likely that involvement of innate immune mechanisms is directive in this process. In the induction phase the innate immunity may prepare the synovium for T cell infiltration and related immune events. Subsequently synovial-lining cells are supposed to be engaged, which produce several chemokines that attract monocytes and lymphocytes into the joint. If these cells recognize an antigen in this environment they will be retained and persisting inflammation may ensue. This hypothesis does not require one specific trigger. Any pathogen that gains entry into the joint could be responsible in a given individual. Furthermore there is a possibility of genetically determined variability in response to the same environmental insult.

The external stimulus initiates T cell-mediated responses directed either against the inciting antigen or against a secondary joint-specific target such as type II collagen. As a consequence the typical clinical picture of RA arises, driven by lymphocytes, antigen presenting cells, and macrophages. As the disease progresses, multiple cytokine networks become established and antigen independent processes become more important. The chronic inflammatory process gradually achieves a certain degree of autonomy of non-antigen specific cells, such as Fibroblast Like Synoviocytes (FLS), which will persist even after down regulation of the initial antigen specific T cell response. In addition to this autonomy of the FLS, experimental models of arthritis support arguments for the existence of a “transformed” phenotype of a fraction of RA synoviocytes. For instance cells from the pannus, directly eroding bone or cartilage, have distinctive morphology and features of both FLS and chondrocytes. These alterations in synoviocyte function might have profound implications for matrix
MMPs in rheumatoid arthritis

destruction, as well as for remodeling and repair in individual joints of individual patients.

II. Pathophysiology of joint destruction in RA

The Extra-Cellular Matrix (ECM) gives structural support to cells and tissues, and plays a central role in cell adhesion, differentiation, proliferation, and migration of its constituents. The turnover of the ECM usually occurs slowly in mature tissues but is accelerated in conditions such as wound healing, arthritic joint destruction, and malignancy.

In the ECM of joints numerous macromolecules have been identified. The major constituents, especially of cartilage, are collagen and the proteoglycan aggrecan, which forms large aggregated complexes with hyaluronan and link protein (figure 1).

FIGURE 1. Schematic representation of aggrecan with its major proteolytic cleavage sites (1,2). The aggrecan molecule has three globular domains (G1, G2 and G3). The N-terminal G1 domain interacts with hyaluronan and a link protein, forming large aggregates. In the interglobular domain between G2 and G3 keratin- (KS) and chondroitin- (CS) sulphate chains are inserted. The two major cleavage sites of human aggrecan in vivo are the “MMP cleavage site”(1) generating the G1-VDIPEN341 and the “Aggrecanase-cleavage site”(2) generating G1-NITEGE373 fragments.

A. Collagen

The collagen meshwork in cartilage contains fibrils of type II collagen and to a lesser extent of collagen type IX and XI, linked together by a number of collagen binding glycoproteins on their surface. Type II collagen consists of 3 identical α chains that form triple-helical monomers that associate in a one-quarter stagger to
form fibrils which also contain type IX and XI collagens. This network provides the cartilage with tensile strength and capacity to resist the swelling pressure exerted by the entrapped water molecules in the proteoglycans 6.

There appears to be little capacity for chondrocytes to regenerate collagen architecture once the mature tissue is injured. Collagen degradation is considered to be a critical and probably irreversible step in cartilage destruction in diseases as osteoarthritis and RA 7.

B. Aggrecan
Aggrecans fill the interstitial spaces of the collagen meshwork by forming large aggregating complexes interacting with hyaluronan and a link protein. Aggrecan is a multidomain glycoprotein composed of a central protein chain, which has 3 globular domains (G1, G2 and G3). In particular the region between G1 and G2 is susceptible to cleavage by members of the Matrix MetalloProteinases (MMPs) and aggrecanases, members of the “A Disintegrin And Metalloproteinase with Thrombospondin Motifs” (ADAMTS) family (figure 1) 8,9. In the interglobular domain between G2 and G3 hydrophylic sulphated glycosaminoglycans (GAGs) such as chondroitin sulphate (CS) and keratan sulphate (KS) chains attract and entrap water molecules, which give cartilage its reversible deforming capacity during joint loading 6.

Proteoglycan degradation is thought to be an early and reversible process, in contrast to collagen degradation, which is believed to be irreversible 9.

C. Joint destruction
The degradation of ECM components of cartilage, bone, tendons and ligaments is a key process in joint destruction in RA, one of the strongest predictors of long term outcome and disability 10. The balance between synthesis of ECM components and activity of destructive proteases as well as their natural inhibitors determines the fate of the ECM.

Cartilage is destroyed by both enzymatic and mechanical processes. Proteolytic enzymes produced by synoviocytes in the pannus, by granulocytes in the synovial fluid, as well as by chondrocytes themselves degrade proteoglycans, such as aggrecan. As soon as cartilage is depleted from proteoglycans it loses its ability to rebound from a deforming load, leading to increased susceptibility to mechanical fragmentation and eventually to loss of functional integrity 11. Besides proteoglycan loss, collagen degradation is an essential and irreversible process in cartilage disruption 9. This destructive process is mediated by several families of proteases such as aspartic, serine and cysteine proteases. However,
the MMPs and aggrecanases, members of the ADAMTS family, are supposed to be key mediators in these processes \(^8,^{12,13}\).

Bone erosion and destruction results mainly from the activation of osteoclasts. RA synovial tissues produce a variety of factors, such as IL-1\(\alpha/\beta\), TNF-\(\alpha\), IL-11, IL-15, IL-17, and others \(^{14}\) with the capacity to increase osteoclast differentiation and activation. In addition activated T lymphocytes and FLS express Osteoclast Differentiation Factor (ODF), also known as Receptor Activator of NF-\(\kappa\)B Ligand (RANKL), critical in inducing the differentiation of cells of the monocyte/macrophage lineage into osteoclasts. Furthermore ODF has the capacity of directly activating multinucleated osteoclasts which are in contiguity with bone \(^{15}\) resulting in resorption of the mineral phase of bone followed by degradation of the remaining matrix by cysteine proteases and MMPs \(^{16}\).

**MATRIX METALLOPROTEINASE FAMILY**

Proteolytic enzymes play critical roles in many biological processes such as growth, development, reproduction, and wound healing. Under normal physiological conditions degradation and synthesis of ECM components are precisely regulated. However, if these processes are disrupted, pathologic conditions may arise \(^7\).

The MMPs are thought to be key enzymes involved in remodeling of the ECM in physiological and pathological situations (e.g. arthritis, cancer, atherosclerosis, and periodontal disease). In diseases as rheumatoid arthritis and osteoarthritis MMPs play a major role in matrix degradation in collaboration with other proteinases, in particular members of the ADAM (A Disintegrin And Metalloproteinase) and ADAMTS (A Disintegrin And Metalloproteinase with ThromboSpondin motif) family also designated as aggrecanases \(^{11}\).

I. **Matrix metalloproteinases**

The MMPs are a family of zinc-containing, calcium-dependent endopeptidases that are active at a neutral pH. Today at least 24 different MMPs have been identified of which 23 are found in humans \(^{18}\). At least 8 of the known human MMP genes are clustered at chromosome 11q21-q23 (MMP-1, -3, -7, -8, -10, -12, -13, and -20). Other known MMPs are scattered among several other chromosomes.

On the basis of their structural and functional properties MMPs are divided into five major groups: I. Collagenases (MMP-1, -8, -13), II. Stromelysins (MMP-3, -10, -11), III. Gelatinases (MMP-2, -9), IV. Membrane-type (MT-) MMPs (MMP-14 to -17, -24, -25), and V. a heterogeneous subgroup (table 1) \(^{19}\).
### Table 1. The matrix metalloproteinases and their substrates

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**I. Collagenases**

<table>
<thead>
<tr>
<th>MMP-1</th>
<th>Interstitial collagenase</th>
<th>Collagens I, II, III, VII, VIII and X, gelatin, aggrecan, versican, proteoglycan link protein, casein, α1-protease inhibitor, α-M pregnancy zone protein, ovostatin, nidogen, MBP, proTNF, L-selectin, proMMP-2, proMMP-9</th>
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<tr>
<td>MMP-8</td>
<td>Neutrophil collagenase</td>
<td>Collagens I, II, III, V, VII, VIII and X, gelatin, aggrecan, α1-protease inhibitor, α2-antiplasmin, fibronectin</td>
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<td>MMP-13</td>
<td>Collagenase-3</td>
<td>Collagens I, II, III and IV, gelatin, plasminogen activator inhibitor 2, aggrecan, perlecain, tenascin</td>
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<tr>
<td>MMP-18</td>
<td>Collagenase-4 (Xenopus)</td>
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**II. Stromelysins**

<table>
<thead>
<tr>
<th>MMP-3</th>
<th>Stromelysin-1</th>
<th>Collagens III, IV, IX and X, gelatin, aggrecan, versican, perlecain, nidogen, proteoglycan link protein, fibronectin, laminin, elastin, casein, fibrinogen, antithrombin-III, α2M, ovostatin, α1-protease inhibitor, MBP, proTNF, proMMP-1, proMMP-7, proMMP-8, proMMP-9, proMMP-13</th>
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<td>MMP-10</td>
<td>Stromelysin-2</td>
<td>Collagens III, IV and V, gelatin, casein, aggrecan, elastin, proteoglycan link protein, fibronectin, proMMP-1, proMMP-8</td>
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<td>MMP-11</td>
<td>Stromelysin-3</td>
<td>α2-protease inhibitor,</td>
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**III. Gelatinases**

<table>
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<tr>
<th>MMP-2</th>
<th>Gelatinase A</th>
<th>Collagens I, IV, V, VII, XI and XIV, gelatin, elastin, fibronectin, aggrecan, versican, proteoglycan link protein, MBP, proTNF, α1-protease inhibitor, proMMP-9, proMMP-13</th>
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<tr>
<td>MMP-9</td>
<td>Gelatinase B</td>
<td>Collagens IV, V, VII, X and XIV, gelatin, elastin, aggrecan, versican, proteoglycan link protein, fibronectin, nidogen, α1-protease inhibitor, MBP, proTNF</td>
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**IV. Membrane-type MMPs**

<table>
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<tr>
<th>MMP-14</th>
<th>MT1-MMP</th>
<th>Collagens I, II, II, gelatin, casein, elastin, fibronectin, laminin B chain, vitronectin, aggrecan, dermatan sulfate proteoglycan, MMP-2, MMP-13, proTNF</th>
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<tr>
<td>MMP-15</td>
<td>MT2-MMP</td>
<td>ProMMP-2, gelatin, fibronectin, tenascin, nidogen, laminin</td>
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<td>MMP-16</td>
<td>MT3-MMP</td>
<td>ProMMP-2</td>
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<tr>
<td>MMP-17</td>
<td>MT4-MP</td>
<td></td>
</tr>
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<td>MMP-24</td>
<td>MT5-MMP</td>
<td>ProMMP-2, proMMP-9, gelatin</td>
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<td>MMP-25</td>
<td>MT6-MMP</td>
<td>Collagen IV, gelatin, fibronectin, fibrin</td>
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**V. Others**

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<tr>
<th>MMP-7</th>
<th>Matrilysin</th>
<th>Collagens IV and X, gelatin, aggrecan, proteoglycan link protein, fibronectin, laminin, entactin, elastin, casein, transferrin, MBP, α1-protease inhibitor, proTNF, proMMP-1, proMMP-2, proMMP9</th>
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<td>MMP-12</td>
<td>Metalloelastase</td>
<td>Collagen IV, gelatin, elastin, α1-protease inhibitor, fibronectin, vitronectin, laminin, proTNF, MBP</td>
</tr>
<tr>
<td>MMP-19</td>
<td></td>
<td>Collagen IV, gelatin, laminin, nidogen, tenascin, fibronectin, aggrecan, COMP</td>
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<tr>
<td>MMP-20</td>
<td>Enamelysin</td>
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<td>MMP-21</td>
<td>XMMMP (xenopus)</td>
<td>Amelogenin</td>
</tr>
<tr>
<td>MMP-22</td>
<td>CMMP (chicken)</td>
<td>Gelatin, casein</td>
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<tr>
<td>MMP-23</td>
<td>CA-MMP</td>
<td></td>
</tr>
<tr>
<td>MMP-26</td>
<td>Matrylissin-2</td>
<td>Collagen IV, fibronectin, fibrinogen, gelatin, α1-protease inhibitor, proMMP-9</td>
</tr>
<tr>
<td>MMP-28</td>
<td>Epilysin</td>
<td>Casein</td>
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In general MMPs are composed of a signal peptide at the amino (NH$_2$) terminal followed by a propeptide domain, a zinc-containing catalytic domain and a haemopexin-like domain at the carboxyl (COOH) terminal as shown in figure 2. The gelatinases (MMP-2, -9) have additional features such as three fibronectin type II repeats in the catalytic domain, that provides them gelatin-binding properties. The MT-MMPs are characterized by a COOH-terminal transmembrane region followed by a short intracytoplasmic tail.

The NH$_2$ terminal signal peptide is a hydrophobic sequence of 8-30 residues, responsible for trafficking of the enzyme through the endoplasmic reticulum and the Golgi apparatus and for its subsequent secretion into the extra cellular space. The signal peptide is cleaved off during the secretion process. The propeptide domain consists of about 80 residues and is responsible for the enzyme latency. It contains one unpaired cysteine in the highly conserved PRCG[V/N]PD motif, the so-called “cysteine switch” (figure 3). In the latent enzyme this sequence of the propeptide is located directly opposite the active site cleft and the SH group of the cysteine interacts with the zinc atom. This prevents the formation of a water-zinc complex that is required for the proteolytic activity. The catalytic domain (about 170 residues) contains the zinc-binding motif HE$xx$H$_{xx}$G$_{xx}$H located 50-55 residues from the COOH terminal in which 3 histidines (H) bind to the

![FIGURE 2](image_url). Schematic representation of the domain structures of the matrix metalloproteinases.
catalytic zinc atom. In addition it contains a conserved methionine, which forms a unique “Met-turn” structure. Between the catalytic domain and the COOH terminal haemopexin-like domain lies a variable stretch of 2-72 amino acids, which is named the “hinge region”. The COOH-terminal haemopexin-like domain of about 200 residues has a number of functions including substrate and inhibitor binding or involvement in activation processes \(^1\)\(^3\),\(^1\)\(^9\). This haemopexin domain is an absolute requirement for the collagenases to cleave triple helical interstitial collagens. As an illustration the amino acid sequence \(^2\)\(^0\) and the crystal \(^1\)\(^9\) structure of MMP-3 are shown in figure 3 and 4.

**FIGURE 3.** Amino-acid sequence and specific sites of matrix metalloproteinase 3. The propeptide is represented in italic and ranges from amino acid 18-99. The “bait region” is involved in the activation of the MMPs (see figure 9).

The secreted collagenases (MMP-1, -8 and -13) and membrane bound MT1-MMP are the only enzymes capable of cleaving the intact triple helix of collagen types I, II, and III. The degradation of the triple helical collagen II occurs at a specific site between residues 775 (glycine) and 776 (leucine) within each \(\alpha\) chain of the triple helical collagen molecule, resulting in the characteristic TC\(^A\) (3/4) and TC\(^B\) (1/4) cleavage products. This opening of the triple helix allows access for other proteinases such as MMP-3, MMP-2, and MMP-9 \(^2\)\(^1\). Besides a role in this process MMP-3 is able to degrade numerous other components of the ECM such as aggrecan, fibronectin, laminin, and several other collagens. And, in addition MMP-3 can activate several pro-MMPs such as pro-MMP-1, -7, -8, -9, and -13.

One of the central pathophysiological features contributing to cartilage destruction is the catabolism and subsequent loss of aggrecan. One well-characterized MMP-cleavage site is the Asn\(^3\)\(^4\)1-Phe\(^3\)\(^4\)2 bond in the interglobular
FIGURE 4. Structure of the catalytic domain of matrix metalloproteinase 3. Strands (sI-sV) and helices (hA-hC) are labelled in black; the catalytic zinc (centre), co-ordinated by three histidine residues (pink), and the structural zinc (Zn) (top) are labelled in black and white, respectively; the NH$_2$ terminus (N) and COOH terminus (C) are labelled in red. The active site cleft and the characteristic methionine (met) turn are indicated by arrows. The figure was adapted, with permission, from Gillian Murphy, Vera Knäuper, Susan Atkinson, George Butler, William English, Mike Hutton, Jan Stracke and Ian Clark: Matrix metalloproteinases in arthritic disease. Arthritis Res 2002, 4 (suppl 3): S39-S49 (reference 19).
http://arthritis-research.com/content/4/S3/S39

domain between G1 and G2 (see figure 1). A cleavage by various MMPs in this region separates a major part of aggrecan (MMP-generated G1-VDIPEN$^{341}$) from its hyaluronan attachment. In 1991 another cleavage site was found at the Glu$^{373}$-Ala$^{374}$ bond in the interglobular domain between G1 and G2 resulting in G1-NITEGE$^{373}$ fragments. The enzyme responsible was referred as "aggrecanase-1", now designated as ADAMTS-4$^{22,23}$.

II. The ADAM(TS)s
The ADAMs are transmembrane proteins. They consist of a NH$_2$-terminal (extracellular) signal peptide, a propeptide (about 170 amino acids), a metalloproteinase domain (about 230 amino acids), a disintegrin domain, a cysteine-rich region usually containing an epidermal growth factor-like domain
and a transmembrane domain followed by an intracytoplasmic tail. The biological functions of many ADAMs are unknown, but for example ADAM-17 also known as tumour necrosis factor α converting enzyme (TACE), is involved in the release of TNF-α as well as of TNF-α receptors and other surface molecules.

The ADAMTSs are related to the ADAMs but they are not membrane-anchored and contain additional thrombospondin type I motifs (see figure 2) responsible for its anchoring to the ECM. The ADAMTS-1, -4, and -5 (the “aggrecanases”) are of major importance in cartilage degradation.

Besides the Glu-Ala bond in the interglobular domain of aggrecan, ADAMTSs cleave several other sites including the MMP-cleavage site Asn-Phe (see figure 1). In arthritic joints MMP-generated G1-VDIPEN and ADAMTS-generated G1-NITEGE fragments are found in cartilage and synovial fluids. There is discussion which group of enzymes plays the primary role in in-vivo cartilage degradation. In in-vitro cartilage explant systems, the initial enzymes responsible for degrading aggrecan are the ADAMTSs followed by MMPs at a later stage.

**REGULATION OF MMP PRODUCTION**

To prevent tissue destruction it is important that the activities of MMPs are tightly controlled. The relative balance between activated MMPs and their inhibitors are thought to determine the rate of ECM turnover. Many MMPs are not constitutively produced but inducible and their production can be regulated at several levels such as signal transduction preceding transcription, transcription itself, or posttranscriptional processing.

**I. Signal transduction pathways**

Transcription can rise dramatically in response to various extra-cellular stimuli such as inflammatory cytokines, growth factors and matrix proteins. These extra-cellular signals are transduced to the nucleus by specific intra-cellular transcription factors generated by signal transduction pathways such as the Nuclear Factor κ B (NF-κB), the Mitogen-Activated Protein Kinase (MAPK) and the Signal Transducers and Activators of Transcription (STAT) pathways (figure 5-7).

For transcription of MMPs several transcription factor binding sites are of importance such as the NF-κB, Activating Protein-1 (AP-1), STAT and Polyoma Enhancer A-binding Protein-3 (PEA-3) sites (figure 8).
A. NF-κB pathway (figure 5)
The NF-κB/Rel family includes NF-κB1 (p50/p100), NF-κB2 (p52/p100), p65 (RelA), RelB and c-Rel. The most important activated forms are a heterodimer of p65 in combination with a p50 or p52 subunit.

Upon binding of IL-1 and/or TNF-α to their respective cell-surface receptors several intracellular proteins are recruited to the cytoplasmic domain of these receptors. These complexes then recruit and activate the NF-κB Inducing Kinase (NIK) and mitogen-activated protein kinase/ERK kinase kinase-1 (MEKK-1), members of the MAPK kinase kinase (MAPKKK) family which activate the Inhibitor of κB Kinase (IKK-α, IKK-β and the regulatory unit IKK-γ). These IKKs phosphorylate the inhibitors of κB (IκBα, -β and -ε), which are the cytosolic inhibitors of "pre-made" NF-κB. After phosphorylation IκB becomes

FIGURE 5. Nuclear Factor-κB (NF-κB) signal transduction pathway. NIK; NF-κB inducing kinase. MEKK-1; mitogen activated protein kinase/ERK (extra-cellular, stimulus regulated kinase) kinase kinase-1. IκKα/β; inhibitor of κB kinase. IκB; inhibitors of κB.
ubiquitinated resulting in proteosome-mediated proteolytic degradation. The p50 and p65 subunits of NF-κB are then translocated to the nucleus where they bind to a NF-κB binding site in promoter regions of MMPs, such as in the MMP-1 promoter region. These binding sites cooperate with other binding sites such as the AP-1 site. This synergistic interaction, "cross-talk" between binding sites, seems to be of importance for the transcription of several MMPs such as MMP-3 and MMP-9.

B. MAPK pathway (figure 6)
Concomitant with activation of the NF-κB pathway IL-1 and TNF-α can induce the MAPK pathway. A MAPK cascade consists of several kinases activating (by phosphorylation) the next kinase, ultimately leading to the activation of transcription factors. For MMP transcription three groups of MAPKs are of importance; a. Extra-cellular stimulus-Regulated Kinases (ERK), b. c-Jun N-terminal Kinases (JNK) and c. p38 MAPK (p38).

After binding of an extra-cellular stimulus, e.g. a cytokine or growth factor to its receptor, small guanylyl triphosphate (GTP)-binding proteins such as Ras (Rac, Cdc42) are activated which in turn activate MAPK kinase kinase (MAPKKK). MAPKKK phosphorylates and activates MAPKK, which activates the MAPK (ERK, JNK, and p38). Once activated the phosphorylated MAPK translocate to the nucleus where they phosphorylate and activate the actual transcription factors such as Erythroblastosis twenty-six (ETS), jun, and Activating Transcription Factor 2 (ATF-2) (part of respectively the ERK-, JNK- and p38-pathway). These transcription factors bind to specific binding sites in gene promoter regions such as the AP-1 site, which binds fos as well as jun families of transcription factors, and the PEA-3 sites, which bind the ETS family. This binding provides the nuclear signal that initiates the transcription of many genes, not only of target genes coding for MMPs but also of genes for transcription factors themselves such as c-fos, jun, and others.

To date the p38 pathway is not completely elucidated. There seems to be no known target of p38 that directly regulates MMP promoters. However p38 can activate transcription factors such as ATF-2 and Elk-1 (a member of the ETS oncogene superfamily) which drive c-jun and c-fos promoters. Possibly by this route of promoting transcription factors which regulate the expression of AP-1 genes, p38 contributes indirectly to MMP transcription.
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C. STAT pathway (figure 7)
Upon binding of an extra cellular stimulus to its receptor Janus Kinases (JAKs) are activated followed by activation of STATs. Once phosphorylated STATs dimerise and translate to the nucleus where they bind to specific transcription response elements in MMP genes $^{28}$. Recently Li and colleagues showed that Oncostatin, a member of the IL-6 superfamily of cytokines induces MMP-1, -3 and -13 and TIMP-3 expression in chondrocytes by activating JAK/STAT and MAPK signaling cascades $^{38}$. 

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FIGURE 6. Mitogen Activated Protein Kinase (MAPK) signal transduction pathway. MEKK; mitogen activated protein kinase/ERK kinase kinase-1. ASK-1; apoptosis signal-regulating kinase 1. ERK; extra-cellular stimulus-regulated kinase. JNK; c-Jun N-terminal kinase. ETS; erythroblastosis twenty six. ATF-2; activating transcription factor-2. PEA-3; polyoma enhancer A-binding protein-3 binding site. AP-1; activating protein-1 binding site.
D. Transcription repression

Transcription can also be inhibited. Glucocorticoids bind to an intracellular glucocorticoid receptor followed by nuclear translocation and binding to glucocorticoid response elements in gene promoters. By this mechanism glucocorticoids can inhibit the expression of multiple inflammatory genes. In addition the glucocorticoid receptor can interfere with the activity of key transcription factors such as NF-κB by activating the IκB-α gene and AP-1 by physical interference with the fos and jun subunits of AP-1.

II. Transcription itself: promoter polymorphisms

Next to these signal transduction pathways Single Nucleotide Polymorphisms (SNPs) in the promoter regions of MMPs seem to be of importance. Promoter regions of many MMP genes contain binding sites for several transcription factors (figure 8).
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FIGURE 8. Promoter region of MMP-3 with transcription factor binding sites. ZBP-89; 89 kDa zinc binding protein. SPRE; stromelysin 1 PDGF responsive element. NF-κB; nuclear factor κB. TCF; T-cell factor. CIZ; Cas-interacting zinc-finger protein. PEA-3; polyomavirus enhancer A binding protein 3. AP-1; activator protein. TBS; TEL (translocation-ETS-leukaemia) Binding site. STAT?: possible binding site of signal transducers and activators of transcription. TATA; TATA box. Reprinted with permission, from Nat Rev Cancer 2002;2(9):657-72 (reference 75), copyright 2002 Macmillan Magazines Ltd.

These elements play important roles in the regulation of MMP transcription both at the basal level and in response to various stimuli including cytokines and growth factors. Naturally occurring sequence variations in the promoters of these genes may result in different levels of expression of MMPs. Approximately 90% of DNA polymorphisms are SNPs due to single base substitutions or insertions. Although the majority is probably functionally neutral, a number of them can exert allele specific effects on the regulation of gene expression of the coded protein.

Promoter polymorphisms have been identified in a number of MMP genes such as MMP-1 and MMP-3. In the MMP-1 gene (locus 11q22-q23) two alleles have been detected, one having a single guanine (1G) and the other having two guanines (2G) at positions -1607 (-1607:1G/2G). The two MMP-3 alleles (at locus 11q23) are defined by the presence of a run of either 5 or 6 adenines at position -1171 (-1171:5A/6A). These polymorphisms have been shown to influence MMP gene expression and are associated with susceptibility and/or severity of diseases such as cancer and atherosclerosis.

In the MMP-1 promoter, at -1607 (and -1608) 2 guanines together with an adjacent adenine can create a core binding site (5'-GGA-3') for the ETS family of transcription factors. This 2G allele binds substantially more ETS-1 and has substantially more transcriptional activity compared to the 1G allele in normal fibroblasts and melanoma cells as well as in ovarian tumour tissue. In the study of Kanamori et al, concerning ovarian tumours, the proportion of 2G carriers was also significantly higher in patients than in healthy controls which might imply an influence on susceptibility. A relation with disease severity was found in a study on malignant melanoma. In this study the 2G allele was associated with deep invasiveness and thereby a worse prognosis.
Studies concerning the -1607:1G/2G MMP-1 polymorphism in RA patients are scarce. In a recent study Constantin et al could not find an association between this SNP in the MMP-1 promoter and the susceptibility to or severity of RA. The MMP-3 promoter polymorphism -1171:5A/6A appears to be functionally important as well. Cultured fibroblasts and vascular smooth muscle cells transfected with constructs containing the run of 6 adenines expressed a roughly 2-fold lower amount of reporter gene product as compared with the transfectants of the constructs containing a run of 5 adenines. Additional studies revealed binding of putative transcription factors to this region. The difference in promoter activity is probably due to preferential binding of a transcription repressor to the 6A allele.

Influences of this polymorphism have been evaluated in atherosclerosis and cancer. The 5A allele was found to be associated with susceptibility of acute myocardial infarction and abdominal aortic aneurysm, diseases associated with weakening of the matrix possibly as a result of increased MMP-3 expression. On the other hand the 6A allele seems to be associated with accelerated growth of coronary atherosclerosis possibly due to accumulation of ECM as a result of an insufficient MMP-3 expression, production, and activation. In breast cancer the presence of the 5A allele seems to be associated with susceptibility and severity. On the other hand in colorectal cancer the 6A/6A genotype was found more often in patients compared to controls.

In RA patients Constantin et al found no association between the -1171:5A/6A polymorphism and disease susceptibility. However, severity and progression, represented by radiologically detectable joint damage, appeared to be associated with the 6A/6A genotype, which is associated with a lower transcriptional activity, in in vitro experiments compared to the 5A genotype. This is somewhat unexpected but possibly linkage disequilibrium is of importance. In the MMP gene cluster the MMP-1 locus is adjacent to the MMP-3 locus and MMP-1 and MMP-3 promoter linkage disequilibrium has been reported. In colorectal cancer the 2G MMP-1/6A MMP-3 haplotype was significantly increased. And in RA patients Keyszer et al showed in a preliminary report that almost all haplotypes were found to be either 1G MMP-1/5A MMP-3 or 2G MMP-1/6A MMP-3.

III. Posttranscriptional regulation

Posttranscriptional regulation (the control of mRNA stability and translation) of pro-inflammatory gene expression is thought to be mediated by the adenosine/uridine rich elements (AREs) within the 3’ untranslated regions of the mRNA flanking the protein coding sequence. AREs have been shown to destabilize
heterologous transcripts into which they are inserted \(^{57}\). Deletion of ARE elements from the TNF-\(\alpha\) locus in the mouse caused an increase in TNF-\(\alpha\) mRNA stability and a loss of the inhibitory effects of specific p38 MAPK inhibitors upon TNF-\(\alpha\) biosynthesis \(^{58}\). A mouse knockout study suggested that the effects of p38 may be mediated by Mitogen Activated Protein Kinase-Activated Protein Kinase 2 (MAPKAPK2) a kinase activated by p38. In absence of the MAPKAPK2 activity, reduced quantities of TNF-\(\alpha\) protein were synthesized whereas the induction of TNF-\(\alpha\) mRNA was unaffected \(^{59}\). Taken together these findings suggest that p38 may regulate TNF-\(\alpha\) mRNA translation by inducing MAPKAPK2 activity and by a mechanism involving the ARE region in the TNF-\(\alpha\) gene. In contrast Brook et al reported that TNF-\(\alpha\) mRNA was clearly destabilized by the p38 MAPK inhibitor SB203580, strongly indicating a role of p38 in the regulation of TNF-\(\alpha\) mRNA stability \(^{60}\). In addition p38 and MAPKAPK2 regulated the stability rather than the translation of IL-6, IL-8, GM-CSF and Cox-2 mRNAs in the human HeLa cell line \(^{61}\). Besides control over mRNA turnover in cytokine and prostaglandin genes, AREs are also required for the regulated decay of MMP-transcripts such as those of MMP-1 and possibly MMP-13 \(^{28}\).

**REGULATION OF MMP ACTIVITY**

I. **MMP activation**

Most MMPs are synthesized as inactive prepro-enzymes and are secreted as inactive pro-enzymes, which have to be activated by proteolytic cleavage. In vivo activating proteases (other MMPs, plasmin, trypsin) attack the so called “bait region” (figure 3 & 9) in the propeptide, resulting in an intermediate peptide with a destabilized cysteine-zinc interaction rendering the final activation site susceptible to a second proteolytic attack usually catalyzed by MMPs but not by the trigger protease \(^{62}\). Some MMPs are activated intracellular such as MMP-11, whereas other MMPs are activated at the cell surface such as MMP-2 \(^{18}\).

Studies concerning the regulation of the ADAMTSs activity are inconsistent and results are probably influenced by species and age of the used tissue and culture conditions. The ADAMTSs possess a furin cleavage site just before the zinc-containing catalytic domain and are therefore probably activated intracellularly and secreted as active enzymes. Further regulation may be transcriptional and/or posttranscriptional at the level of mRNA stability and/or translation \(^{8}\).
II. MMP inhibition

Activated MMPs can be inactivated by inhibitors such as the Tissue Inhibitors of MetalloProteinases (TIMPs) or α-2 macroglobulin (α-2M). Whereas α-2M acts primarily as a regulator in the systemic circulation, the TIMPs are considered to be the key inhibitors locally in the tissues. TIMPs are 2 domain molecules, consisting of an N-terminal domain of about 125 amino acids and a smaller C-terminal domain of about 65 residues, each domain being stabilized by three disulfide bonds. The shape of the TIMP molecule is like a wedge, which slots into the active-site cleft of an MMP in a manner similar to that of the substrate. Although the four different TIMPs bind to most activated MMPs by forming a 1:1 enzyme-inhibitor complex, significant differences have been reported between different TIMPs. For example TIMP-3 is a good inhibitor of TACE and seems to be the most important inhibitor of ADAMTS-4 and -5. Besides inhibition of activated MMPs, TIMPs are important for pro-MMP activation (pro-MMP-2), cell growth promotion, inhibition of angiogenesis, and induction of apoptosis (reviewed by Brew et al).

α-2M is a plasma glycoprotein synthesized by the liver and by macrophages and fibroblasts. It is a large (M_r 725,000) plasma protein and responsible for inhibition of activated proteases in the systemic circulation. α-2M is active against many endopeptidases regardless of their specificity. The activated protease attacks α-2M in the “bait-region”. This triggers a conformational change in the α-2M molecule, which in turn entraps and inactivates the enzyme. Subsequently the α-2M-proteinase complexes are rapidly cleared by macrophages and fibroblasts by receptor-mediated endocytosis.

![Figure 9: Stepwise activation of proMMPs. Zn; zinc. C; cystein. Cleavage sites are indicated by an arrow (↑ ↓)](image-url)
III. Balance
Under physiological conditions the levels of protease inhibitors exceed the levels of their targeted proteases. Controlled activation of MMPs and inhibition by local inhibitors such as TIMPs will allow a low levels of turnover to occur. Many proteases, including MMPs are not constitutively produced but inducible and in pathological conditions transcription, translation and activation can increase substantially. In contrast, protease inhibitors are constitutive proteins of which the production can also be increased by cytokines such as IL-1, IL-6 and TNF-α. Given the important role of MMPs in tissue destruction, the relative balance between activated MMPs and their inhibitors such as TIMPs and α-2M will ultimately determine the fate of the ECM.

ROLES OF MMPs
I. Physiologic
Because MMPs can degrade almost all components of the ECM their main function is presumed to be ECM remodeling e.g. during embryonic development, tissue growth, and morphogenesis. In fact the discovery of the MMPs in 1962 was based on the observation that during amphibian metamorphosis a collagenolytic activity had to be present to digest the collagens in tadpole tails.\(^6^7\) Ongoing research has shown that MMPs not only remodel the ECM but may also be important in a number of other physiologic processes: a. MMPs may affect cell migration by changing cells from an adhesive to a non-adhesive phenotype and/or by ECM degradation, b. MMPs may alter the micro-environment of the ECM leading to alterations in cell behaviour (proliferation, differentiation, organization, survival), c. MMPs may modulate the activity of growth factors or their receptors by releasing them from the ECM, and d. MMPs themselves may alter the balance of other protease activities by cleaving these enzymes and/or their receptors.\(^6^8\) It has to be emphasized that most data concerning the roles of MMPs are derived from in-vitro experiments. In vivo experiments may well yield different results implicating compensatory mechanisms.

II. Pathologic
The roles of MMPs have been described in many diseases associated with an unbalanced degradation of ECM such as in rheumatic diseases but also in non-rheumatic diseases such as cardiovascular disease, cancer, lung-, inflammatory bowel-, and renal disease.
A. Non-rheumatic diseases
Cardiac and arterial remodeling by MMPs occurs in various cardiovascular diseases such as abdominal aortic aneurysms, atherosclerosis, and heart failure. Several preclinical studies with MMP-inhibitors in animal models with heart failure have shown less damage of matrix collagen, favourable matrix remodeling, and improved cardiac structure and function.

In tumour invasion at least three steps are of importance. First the affinity of cells either for each other or for the ECM should decrease in order to allow cell release from the primary tumour. Second, the surrounding ECM should be remodeled by the production of proteolytic enzymes in order to allow the third step, cell migration. There is considerable evidence to suggest that MMPs are not only involved in this local invasion but also in tumour growth (by altering the local environment, e.g. by allowing the access or release of growth factors into or from the ECM) and in the metastatic process. The increased MMP-expression in host stromal cells rather than in tumour cells themselves underlines the importance of tumour-stroma interactions.

In view of the role of MMPs in cancer the inhibition of their activity is a novel target for the treatment of malignancies. Several synthetic MMP-inhibitors have been developed and tested in phase I, II, and III clinical trials and have been reviewed elsewhere.

In various other pathological conditions the MMPs seem to play important roles such as in lung disease, neurological diseases, and in gastrointestinal disease.

B. Rheumatic diseases, non-RA
Due to the important role of MMPs in matrix remodeling and degradation, many studies have been reported in rheumatoid arthritis and to a lesser extent in other rheumatic conditions such as osteoarthritis, SLE, scleroderma, psoriatic arthritis, ankylosing spondylitis, crystal arthropathies, etc. In many of these studies systemic levels of MMPs were evaluated, in particular MMP-3.

Osteoarthritis (OA) is a slowly progressive disorder characterized by destruction of joint cartilage and subchondral bone, with “mild” inflammatory alterations. The MMPs are thought to play an important role in the degradation of the ECM in OA. Several studies have shown an increase in MMP gene expression and MMP protein production as well as in collagenase-mediated type II collagen degradation products in tissues of OA patients. In synovial fluid of the knee of patients with OA several MMPs can be detected with a predominance of MMP-1, MMP-2, and in particular MMP-3. In the
systemic circulation of OA patients MMP levels are generally low or undetectable. Some studies report elevated systemic MMP-3 levels \cite{87-89} whereas other investigators found no differences compared to healthy controls \cite{90,91}. In a subgroup of patients with rapidly destructive osteoarthritis of the hip elevated systemic levels of MMP-3 and MMP-9 have been reported \cite{92}. Although there is consensus that these enzymes do contribute to the degenerative changes, their relative role is still uncertain \cite{85}.

In SLE systemic MMP-3 levels have been reported to be elevated but serial measurements did not reveal a relationship to disease activity \cite{93}. On the other hand in a study of Kotajima et al, patients with clinical and serological features of lupus nephritis had significantly increased serum MMP-3 levels \cite{94}. Since arthritis is less frequent in SLE MMP-3 might be produced extra-articularly, possibly in the kidney.

In scleroderma both normal and elevated systemic MMP-1 and MMP-3 levels have been reported. In most studies TIMP-1 levels were increased \cite{95,96}. This suggests that tissue matrix accumulation in scleroderma may be due to increased levels of inhibitors rather than to decreased MMPs \cite{73}.

In several other rheumatic diseases such as psoriatic arthritis \cite{91,97}, ankylosing spondylitis \cite{97}, and crystal arthritis \cite{89,91} elevated systemic MMP-3 levels have been reported mainly in view of the comparison with levels primarily determined in RA patients.

**MMPs in rheumatoid arthritis**

**I. Introduction**

RA is characterized by chronic inflammation of synovial tissue and in most cases progressive destruction of cartilage and bone \cite{98}. Progressive joint destruction is one of the strongest predictors of long-term outcome and disability in RA \cite{10}. The MMPs are considered to be key mediators in this destructive process. The principle targets of destruction are interstitial collagens, and secreted collagenases (MMP-1, -8 and -13) have a major role in this process \cite{37}. These three collagenases are upregulated and found locally in inflamed synovial tissue \cite{99,100}, chondrocytes \cite{101}, or synovial fluid \cite{86} of inflamed joints. Besides these collagenases, MMP-3 has been mentioned as an important mediator in matrix degradation \cite{102}.

**II. Role of MMP-3**

MMP-3 is thought to play a prominent role in the pathogenesis of matrix degradation \cite{12,17,102} even though it is not the only key enzyme \cite{103}. MMP-3 is
capable of degrading many components of the matrix in the synovial joint including proteoglycans, gelatin, laminin, fibronectin, and collagen III, IV, and IX. Moreover, MMP-3 is able to activate other matrix metalloproteinases such as MMP-1, MMP-7, MMP-8, MMP-9, and MMP-13.

MMP-3 is produced abundantly in the inflamed joints of RA patients. The enzyme has been localized in the fibroblast-like synoviocytes of rheumatoid synovium, in RA cartilage, at sites of cartilage erosion, in synovial fluid, and in the systemic circulation. MMP-3 is locally produced and activated within the inflamed joint and released into the synovial fluid and systemic circulation. Systemic MMP-3 levels are considered to be a reflection of local synthesis. As such, serum MMP-3 can be used as a systemic marker of local joint inflammation and/or destruction. Therefore serum MMP-3 may reflect joint inflammation and destruction more directly compared to C-reactive protein (CRP) which is produced indirectly by the liver after cytokine stimulation. This difference in marker characteristics can be of importance for new therapies. For example, specific MMP-inhibitors may uncouple the relation between surrogate markers of joint inflammation (such as CRP) and joint damage. Radiological progression might be stopped by such agents without inhibition of inflammation and the acute phase response. In that case new markers of joint destruction, such as MMPs may become essential.

III. Future perspectives, MMP-inhibition
Despite optimal care and recent new therapeutic advances in RA, such as TNF-α blocking agents and IL-1 receptor antagonist, it is often not possible to completely stop joint destruction. In RA and OA progressive destruction of cartilage and bone is considered to be a consequence of a dysbalance between an excess of activated MMPs and inadequate levels of their inhibitors. Therapeutic interventions might be focused on inhibition of the synthesis and release of proMMPs, inhibition of the activation of proMMPs, inhibition of activated MMPs, stimulation of TIMP synthesis or inhibition of TIMP inactivation. One of the key requirements in developing specific MMP-inhibitors is to determine in vivo the importance of individual MMPs in the pathogenesis of arthritis. Two experimental approaches have been used: knockout and transgenic mouse technologies and effects of MMP-inhibitors in cultured cells and animal models.

The MMP-3 knockout mice show a normal development and demonstrate in the collagen-induced arthritis model, similar severity of arthritis and cartilage

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MMPs in rheumatoid arthritis
degradation as wild type animals. MMP-2 deficient mice also develop normally but display reduced angiogenesis and tumour progression. In an antibody-induced arthritis model the MMP-2 knockout mice exhibited both a clinically and histologically severe arthritis when compared to wild type mice or MMP-9 deficient animals. MMP-9 deficient mice exhibit an abnormal vascularization and ossification of the skeletal growth plate but will eventually develop normally. The mild phenotypes of these knockout mice suggest that these individual MMPs (MMP-2, -3, -7, -9, and -12) are not critical during normal development and can be compensated for by other MMP family members.

The MT1-MMP knockout mice display a severe phenotype with dwarfism, osteopenia and arthritis, which suggests severe side effects to be expected in case of specific inhibition.

In a transgenic mouse model overexpression of a constitutively active form of the MMP-13 gene induced cartilage erosions resembling human OA due to collagen and proteoglycan degradation.

In addition to these knockout and transgenic studies both broad-spectrum and more specific MMP-inhibitors have provided insight into the role of individual MMPs in joint destruction. The importance of collagenases in RA and OA has been demonstrated with Ro-32-3555 (Trocade®) a compound, which "selectively" inhibits MMP-1, -8, and -13. In a Propionibacterium acnes rat model Ro-32-3555 did block cartilage degradation despite continuing synovitis. In the STR/ORT mouse model of OA it effectively inhibited cartilage degradation, joint space narrowing and osteophyte formation. These data provide evidence for an important role of MMPs in joint destruction and the potential effects of MMP-inhibitors. However, it also highlights the fact that these compounds may not affect the inflammatory process itself, which would have to be controlled by additional treatments.

Over the last 10-15 years a number of MMP-inhibitors have been developed. In addition to the rheumatic diseases, the main therapeutic focus has been directed to the treatment of cancer. The different compounds have been extensively reviewed elsewhere. Treatment strategies are now focused on inhibition of MMP activity and/or MMP gene expression.

A. Inhibition of MMP activity
Originally MMP-inhibitors were designed to mimic peptide sequences surrounding the point in the collagen molecule first cleaved by collagenases. These “peptomimics” fit into the active sites of MMPs and chelate the zinc atom.
through a zinc-binding group. A major drawback of the approach is that these agents inhibit a broad spectrum of MMPs and are therefore likely to cause unwanted side effects. For example Marimastat®, a broad spectrum MMP-inhibitor has been shown to improve the survival of patients with gastric cancer but the drug caused musculoskeletal pains, mainly in shoulders and hands as a side effect, possibly caused by inhibiting physiological levels of other MMPs.

Despite great efforts in this therapeutic approach the applicability of MMP inhibition as treatment of RA or OA is still undetermined. Ro-32-3555 (Trocade™), a hydroxamic acid that “selectively” inhibits the collagenases and has little activity against other MMPs is the only MMP-inhibitor having completely entered clinical trials to assess efficacy. However, despite good preclinical data the radiographic scores of patients with RA did not differ from placebo and the study was terminated.

Currently the only MMP inhibitor on the market is the tetracycline periostat, which is licensed for periodontal disease. Tetracyclines are able to suppress MMP activity in e.g. adjuvant arthritis. In patients with joint disease a significant reduction in MMP activity within the joints is observed after oral minocyclin or doxycycline. Clinical trials with tetracyclines in RA show promising results with respect to disease activity but failed to show clear effects on radiological progression. Insufficient dosing and residual proteolytic activity, not inhibited by tetracyclines, are reported as possible explanations.

In addition several other issues are of importance. In the first place almost all “specific” MMP inhibitors do not influence the inflammatory components of a disease such as RA. Consequently patients still require their background drug treatment. This might have consequences, not only for compliance (the patient feels no short-term benefit), but also for the evaluation of additional effects of MMP-inhibitors. Differences between groups will become smaller, especially when more effective therapies such as anti-TNF-α are used. Probably large long-term trials will be necessary with patients on a ”stable” DMARD regime, with prescribing guidelines, and stratification.

Secondly it is of importance to consider the outcome and the assessment of the outcome. Although prevention of radiologically detectable damage is an established goal, there is still the question how to translate this into clinical benefit for the individual patient in terms of disability. Furthermore new DMARDs, such as anti-TNF-α are very successful in inhibiting radiological progression in many cases. In that situation an additional effect of a specific MMP-inhibitor is difficult to detect.
MMPs in rheumatoid arthritis

In the third place safety issues have already been mentioned. Several broad-spectrum MMP inhibitors were complicated by musculoskeletal pains, probably due to inhibition of other MMPs. Moreover, because of the role of MMPs in physiological processes such as embryonic development, tissue growth, and morphogenesis MMP inhibitors are likely to be teratogenic.

B. TACE (TNF-α converting enzyme)
Another type of inhibition of MMP activity is the interference with TACE. This enzyme, an ADAM, releases the soluble TNF-α from its membrane and thereby enables the cytokine to act in a paracrine and endocrine manner. TACE is over-expressed in synovial tissue of patients with RA and its inhibition has been shown to be efficacious in experimental arthritis. Phase I studies, of TACE inhibitors have been performed and results from Phase II studies are awaited.

C. Inhibition of MMP gene expression
Due to the difficulties encountered with MMP active site inhibitors, other approaches are currently evaluated. A more selective inhibition of inducible pathological gene expression, without affecting constitutive physiological gene expression is such a line. In particular genes coding for cytokines, prostaglandins and MMPs are of interest. Selective inhibition of inducible MMP genes in RA without affecting constitutive, physiological MMP expression, could have major advantages with regard to potential side effects.

Recent studies have focused at the signal transduction pathways. Blockade of the MAPK and/or NFκB pathways leading to inhibition of MMP expression are effective in tissue culture experiments and in animal models of arthritis. Clinical studies targeted at signal transduction pathways are currently evolving.

By the way, since multiple steps and pathways contribute to MMP synthesis it is important to emphasize that therapies targeted at one step may be only partially effective. Moreover, many intracellular signaling enzymes are not completely specific for one signaling cascade, a phenomenon which bears the risk of a broader spectrum of side effects.

Non-specific inhibitors of MMP gene expression are already used in the treatment of arthritis. Corticosteroids block gene transcription by direct binding to glucocorticoid responsive elements in gene promoters or by interference with transcription factors such as NFκB and AP-1. However, corticosteroids are not specific. They inhibit transcription of many other genes and the frequently occurring side effects associated with these drugs suggest the need for more
specific MMP gene expression inhibitors.

**D. Biologicals**

Another way to reduce MMP levels is by direct blockade of inflammatory cytokines such as TNF-α\(^{144-146}\). Anti-TNF-α therapies reduce inflammation and presumably also reduce MMP synthesis, resulting in reduced joint destruction\(^ {146-148}\). Radiological progression is even absent in infliximab treated patients without a clinical response\(^ {147}\), an argument for a possible dissociation of the two pathophysiological mechanisms of joint inflammation and articular destruction\(^ {149}\). In the future, the concept of combination therapy may become more “evidence based” focused on specific signal transduction pathways or genes. In that context it is essential to realize that further research with respect to markers of disease activity and destruction is of great importance. Not only because specific MMP inhibition might result in an uncoupling of inflammation and destruction but also because currently used parameters of inflammation such as the acute phase proteins such as CRP are mediated by similar signal transduction pathways\(^ {150}\).

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REFERENCES


Chapter 2


Chapter 2


