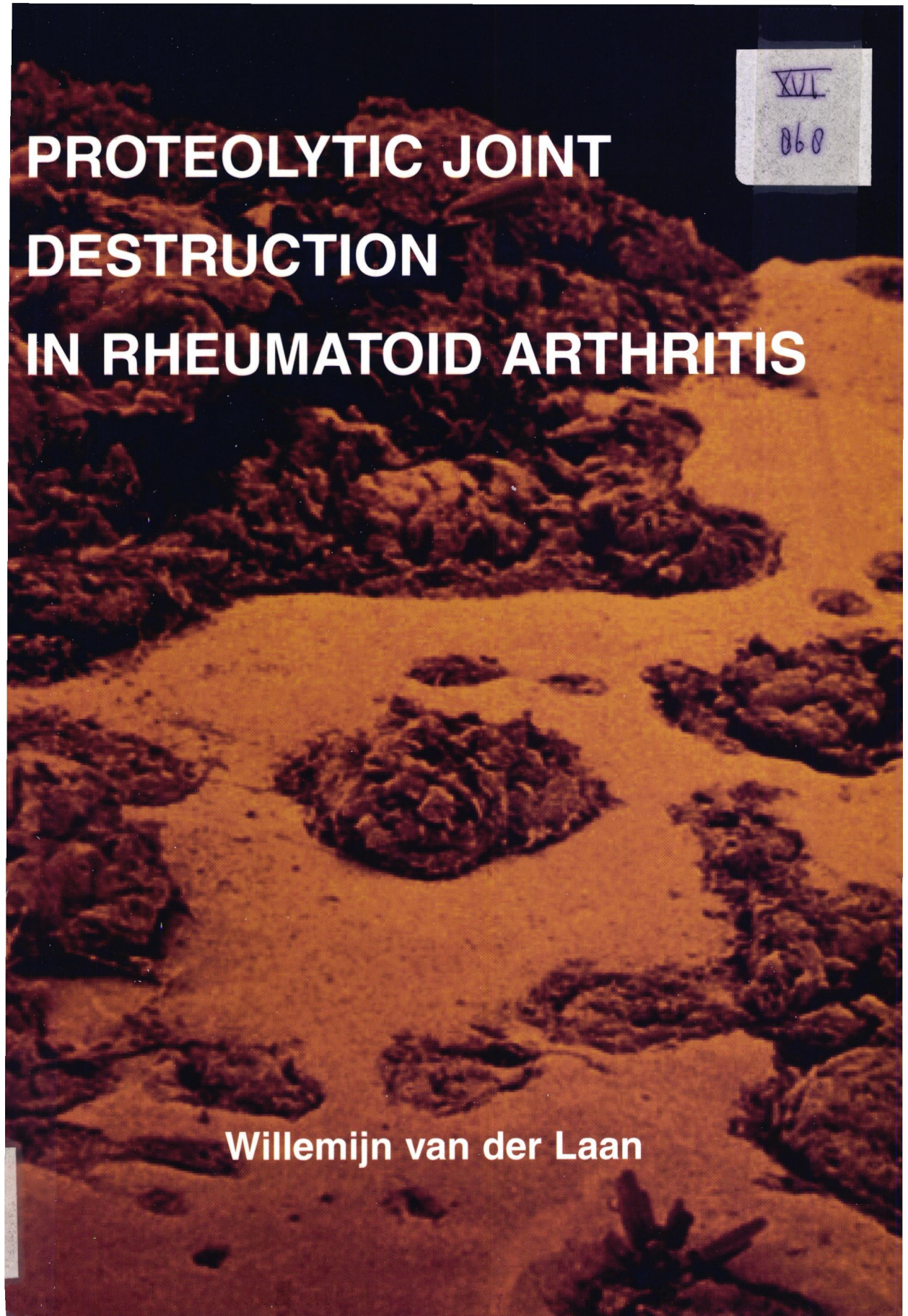
PROTEOLYTIC JOINT DESTRUCTION IN RHEUMATOID ARTHRITIS



Willemijn van der Laan

PROTEOLYTIC JOINT DESTRUCTION IN RHEUMATOID ARTHRITIS

PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus Dr. D. D. Breimer,
hoogleraar in de faculteit Wiskunde en
Natuurwetenschappen en die der Geneeskunde,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 7 februari 2002
te klokke 14.15 uur

door

Willemijn Henrieke van der Laan
geboren te Rotterdam in 1970

Promotiecommissie

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The studies presented in this thesis were performed in Leiden at the Gaubius Laboratory of TNO Prevention and Health and the Department of Rheumatology of the Leiden University Medical Center.

Financial support by the Gaubius Laboratory of TNO Prevention and Health, the Dutch Arthritis Association, the J.E. Jurriaanse Stichting, the Dr.Ir. van de Laar Stichting, and Wyeth / AHP Pharma BV for the publication of this thesis is gratefully acknowledged.

ISBN 90-77017-41-0

STELLINGEN

behorende bij het proefschrift
Proteolytic Joint Destruction in Rheumatoid Arthritis

1. Reumatoïde synoviale fibroblasten invaderen met behulp van plasmine en MMPs het gewrichtskraakbeen. (Dit proefschrift)

2. De bevinding, dat het afzonderlijk remmen van plasmine en MMPs in hetzelfde model de invasie van kraakbeen door reumatoïde synoviale fibroblasten remt, betekent niet noodzakelijkerwijs dat plasmine en MMPs onderdeel zijn van hetzelfde afbraakmechanisme. (Dit proefschrift)

3. De destructie van kraakbeen door reumatoïde synoviale fibroblasten wordt beïnvloed door factoren die kraakbeencellen uitscheiden. (Dit proefschrift)

4. Bij patiënten met reumatoïde artritis is een systemische behandeling met de plasmineremmer tranexaminezuur of MMP-remmer doxycycline geen effectieve therapeutische benadering om gewrichtsdestructie te remmen. (Dit proefschrift)

5. De effectiviteit van een enzym dat kraakbeendestructie door reumatoïde synoviale fibroblasten remt neemt toe wanneer de enzymremmer wordt geconcentreerd op de plaats van destructie, bijvoorbeeld door te binden aan het celoppervlak. (Dit proefschrift)

6. Bij reumatoïde artritis is de pathogenese van erosies niet direct gekoppeld aan de aanwezigheid van synovitis. (Cunnane et al. *Arthritis Rheum* 2001;44:2263-74; Kirwan et al. *Br J Rheumatol* 1997;36:225-8)

7. De aanwezigheid van fibrine deposities in het synovium is een niet-immunologische factor die bijdraagt aan het in stand houden van de chronische gewrichtsontsteking bij patiënten met reumatoïde artritis. (Busso et al. in *J Clin Invest* 1998;102:41-50)

8. De bevinding dat deficiëntie van MT1-MMP, in tegenstelling tot andere MMPs, grote gevolgen heeft voor de ontwikkeling van het skelet en bindweefsel van muizen suggereert dat dit enzym een sleutelrol vervult in de processen van weefselombouw. (Holmbeck et al. in *Cell* 1999;99:81-92)

9. Gerandomiseerde klinische onderzoeken met externe sponsoring vertonen vaker significant positieve resultaten dan trials zonder sponsoring. (Davidson RA. *J Gen Int Med* 1986;1:155-158)

10. De acceptatie van een nieuwe theorie of behandelwijze wordt niet zo zeer bepaald door statistisch significante resultaten, maar vooral door de vraag of deze past in de bestaande dogma's. (Naar J.P. Vandenbroucke, *Gezonheidsraadlezing* 1999)

11. De term "evidence-based medicine" is misleidend, omdat de bewijzen waarop deze gebaseerd is onderhevig zijn aan publicatie-, interpretatie- en sponsorbias.

12. Hoe sneller de computer, hoe ongeduldiger de gebruiker.

13. "Artsen moeten niet alleen de gezondheid herstellen, maar moeten ook de pijn en de ellende wegnemen. En niet alleen als dat tot herstel kan leiden, maar ook om onze laatste gang makkelijker en rechtvaardiger te maken." (Francis Bacon in *Atlantis,* 1627)

Willemijn van der Laan
Leiden, 7 februari 2002

"Ware kennis is weten tot hoever je onwetendheid reikt"

Confucius (*Analects*, 6e eeuw v.Chr.)

CONTENTS

1

GENERAL INTRODUCTION

CONTENTS

1. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting about 1% of the population. The inflammation is primarily located in the synovial joints. However, RA is not confined to the joints and is therefore considered a systemic disease. Common extra-articular manifestations are nodules, serositis, eye involvement, Sjögren's syndrome, and vasculitis.
Many patients with RA present with swelling, pain, and limited motion of multiple joints. The vast majority of the patients develops joint deformities as a result of progressive destruction of the articular cartilage and bone (Figure 1). In early RA, the disease activity mainly determines the impairment of functional capacity. In later stages of the disease, joint destruction becomes an increasingly important determinant of disability.[1,2]

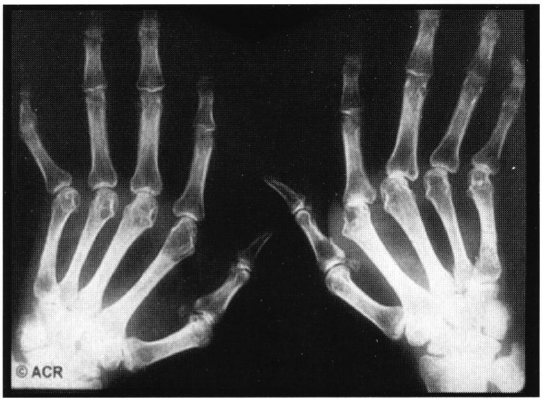

Figure 1. Radiographs of the hands of a patient with RA. *The progressive destruction of the articular cartilage and bone lead to deformations of the joints.*

What exactly initiates the synovitis in RA is still unknown, but it is generally accepted that immune-mediated mechanisms are crucial in the pathogenesis of RA. Several factors that contribute to the susceptibility for the disease have been identified. Twin studies show that genetic factors are involved, since 12-20% of identical twins develop RA compared with 0-3.5% of fraternal twins.[3-5] Women are approximately three times more affected than men,[6] which may suggest that hormonal factors also influence the susceptibility for RA. Since genetic and hormonal factors only partially explain the susceptibility for RA, it is likely that environmental factors also contribute to the pathogenesis of RA. Infectious agents have been studied extensively for their possible role in the pathogenesis of RA, but until now solid proof for a role of any infectious agent is lacking.[7]

2. The normal joint

RA affects diarthrodal synovial joints. The normal joint consists of two bones covered with cartilage that are connected by a capsule. The non-cartilaginous surfaces of the synovial joints are lined by the synovium, a membrane-like tissue between the fibrous joint capsule and the fluid-filled synovial cavity (Figure 2).

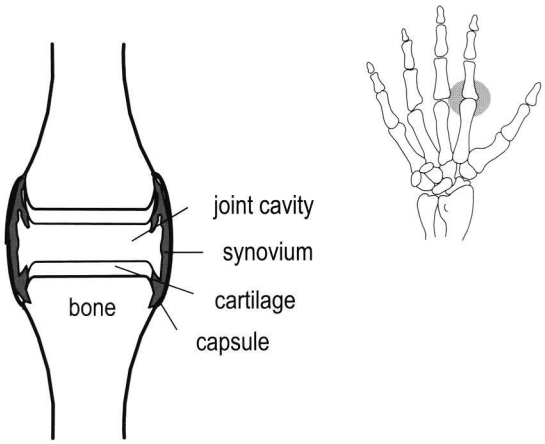

Figure 2. Schematic representation of a normal proximal interphalangial joint. *The synovium is a thin membrane and the articular cartilage and bone are intact.*

The articular cartilage is an avascular tissue that depends for its nutrition on diffusion from its surrounding tissues. Chondrocytes produce and maintain the extracellular matrix of the cartilage, consisting of collagen, proteoglycans and, to a lesser extent, of non-collagenous proteins and non-proteoglycan molecules. (Figure 3A). Articular cartilage has to be resilient to compression, but at the same time has to be able to undergo limited deformation in order to distribute compressive forces over the articular surfaces. Both collagen and proteoglycans contribute to these load-bearing properties. Because proteoglycans are negatively charged, they attract water, resulting in a 65 to 80% water content, which gives cartilage its resistance to compressive forces. The collagen network provides a framework in which the proteoglycans and chondrocytes are embedded and imparts tensile strength to the cartilage.[8,9]

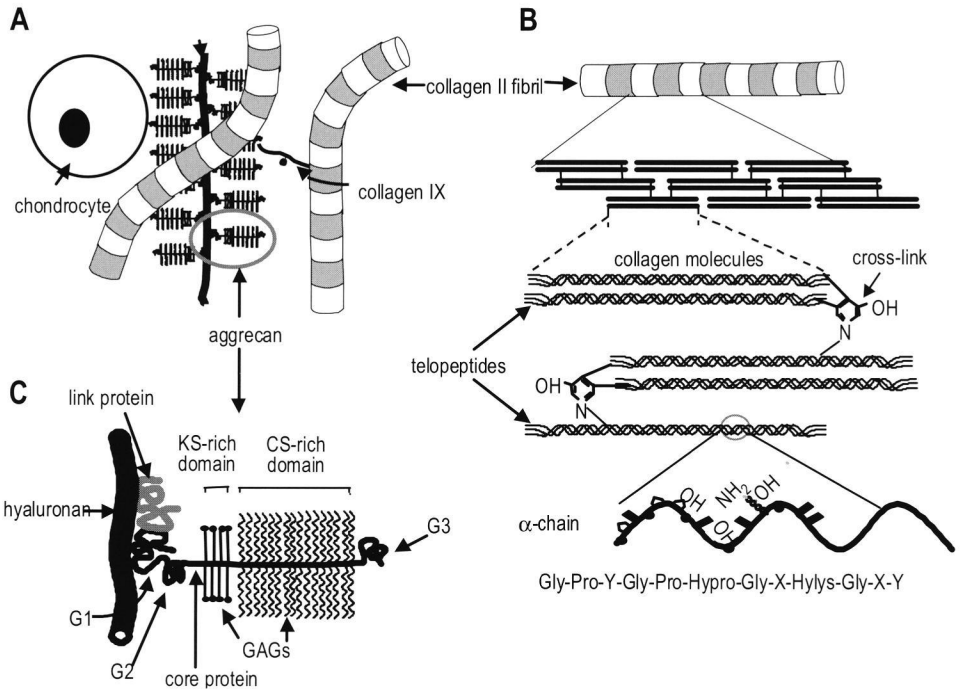

Figure 3. Composition of articular cartilage. **A**. *Articular cartilage is built up by chondrocytes and macromolecules, particularly collagens and large aggregates of proteteoglycans*. **B**. *Collagen fibrils are built up by collagen molecules that are connected to each other by cross-links, such as hydroxylysylpyridinoline (HP). Collagen molecules are formed by a triple helix of α-chains. The α-chains are built up by series of triplets of aminoacids with glycin (Gly) at every third place (Gly-X-Y). X is frequently proline (Pro) and Y is frequently hydroxyproline (Hypro).*

C. *The predominant proteoglycan in articular cartilage is aggrecan. By interaction of the first globular domain (G1) and the link protein with hyaluronan, large aggregates of aggrecan monomers are formed. Glysaminoglycans (GAGs) form highly negatively charged side chains to the core protein. Aggregan contains keratan sulfate (KS)-rich domains and chondroitine sulfate (CS)-rich domains.*

2.1. Macromolecules of articular cartilage

2.1.1. Collagens

Collagen accounts for the major proportion of the dry weight of articular cartilage (50 to 90)[8] Collagens are large proteins that are composed of three polypeptide chains (α-chains). The α-chains are built up by series of triplets of aminoacids with glycin at every third place (Gly-X-Y). This allows the folding of the α-chains into a triple-helix structure. X is frequently proline and Y is frequently hydroxyproline. Hydrogen bonds between the α-chains stabilize the triple-helix (Figure 3B). More than 15 different kinds of collagen have been described and have been divided into four subclasses: 1) fibrillar collagens, 2) basement-membrane-associated collagens, 3) fibril-associated collagens, and 4) short chain collagens.[10] The triple helices of fibrillar collagens (type I, II, and III) have linear peptide extensions called telopeptides. By cross-linking the different triple helices at the site of the telopeptides, rope-like collagen fibrils are formed. This fibrillar structure gives collagen its tensile strength. Collagen type I is found in many connective tissues including bone, ligaments and skin. Collagen type II is specific for cartilage. Type IX and XI are fibril-associated collagens. These fibril-associated collagens are probably important in the formation of a network of collagen fibrils by forming bridges between the collagen fibrils.

2.1.2. Proteoglycans

Proteoglycans are a diverse group of complex macromolecules that are composed of a linear core protein with three globular domains interspersed by an extended linear region to which long-chain polysaccharides, glycosaminoglycans are covalently bound (Figure 3C). The predominant proteoglycan in articular cartilage is aggrecan. The glycosaminoglycan side chain is a highly negatively charged unbranched repeating dimeric polysaccharide, of which three types are found in aggrecan: chondroitin–4-sulfate, chondroitine-6-sulphate, and keratan sulfate.[11,12] The first globular domain (G1) interacts with hyaluronan forming highly charged aggregates, which are responsible for pulling water into the tissue providing the cartilage with the resistance to compressive forces. A link protein with a high homology to the G1 domain of aggrecan stabilizes the interaction of the core protein of aggrecan to hyaluronan.

Other proteoglycans in articular cartilage are the small, leucine-rich proteoglycans, such as decorin, fibromodulin, and biglycan. These molecules bind to collagen fibrils and probably contribute to the stability of the collagen network.[13]

2.1.3. Non-collagenous proteins and non-proteoglycan molecules.

Several non-collagenous, non-proteoglycan molecules form a small proportion of the articular cartilage. For example, cartilage oligomeric protein (COMP) is a homopentamer of five

subunits that can interact with helical collagen. The function of COMP may be to stabilize the collagen network and/or to promote the collagen fibril assembly.[13]

2.2.The synovium

The synovium is divided into functional compartments: the lining region (synovial intima), the subintimal stroma, and the vasculature[9]. Under normal conditions the synovial intima consists of only one to three layers of cells. There are several types of lining cells: 1) macrophage-derived type A synoviocytes, 2) fibroblast-derived type B synoviocytes, and 3) bone marrow cells. The predominant cells in normal synovial intima are the synovial fibroblasts; only ten to twenty percent of the cells are macrophages. Like other fibroblasts, synovial fibroblasts synthesise extracellular matrix and have the potential to proliferate. The subintimal stroma is a cellular (predominantly fibroblasts), well-vascularized tissue that becomes increasingly fibrous when it blends with the joint capsule.[9]

3. The rheumatoid joint

The primary target in RA is the synovium. In chronic stages the synovium is hypertrophied and edematous. Increased numbers of intimal macrophages[14,15] and synovial fibroblasts[16,17] accumulate in the synovial intima. In the subintimal stroma vascular proliferation is present[18,19] with variable numbers of inflammatory cells including macrophages,[14,18] T-lymphocytes, B-lymphocytes, plasma cells, dendritic cells, and neutrophils.[20] The hypertrophied synovium adjacent to the articular cartilage develops into a destructive tissue structure, called pannus. The pannus invades and erodes the articular cartilage, subchondral bone and ligaments, finally leading to deformations of joint (Figure 4). The abundance of fibroblast- and macrophage-derived factors in the rheumatoid synovium implicate macrophages and fibroblasts as effector cells in the chronic stage of RA.[14,16,20,21] Both cell types secrete various cytokines and tissue degrading enzymes. The synovial macrophages are the main source of inflammatory mediators such as tumor necrosis factor (TNF)-α and interleukin -1 (IL-1)-β, which stimulate the synovial fibroblasts to secrete tissue degrading enzymes as well as inflammatory mediators.[14,16] The tissue degrading enzymes (discussed below) degrade the articular tissues causing progressive destruction of joints[22].

In addition to the synovial lining cells, chondrocytes and osteoclasts also contribute to joint destruction in RA. Chondrocytes digest the matrix in which they are embedded in response to proinflammatory cytokines such as IL-1-β and TNF-α.[23,24] Articular bone is mainly degraded by osteoclasts in response to factors that stimulate osteoclastogenesis and bone resorption.[25-28]

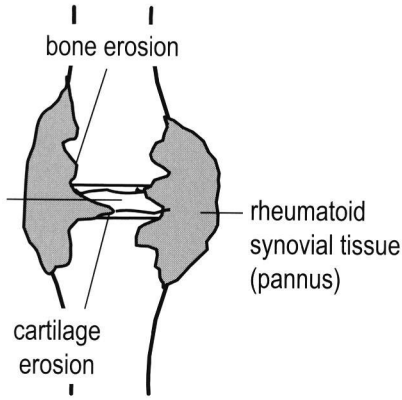

Figure 4. Schematic representation of a joint of a patient with destructive RA. *Invasion of the rheumatoid synovial tissue (pannus) into the articular cartilage and bone results in loss of the cartilage, visual on a radiograph as narrowing of the joint space, and bony erosions.*

3.1. The RA synovial fibroblast

Several properties of rheumatoid synovial fibroblasts that may contribute to the pathogenesis of synovitis and joint destruction in RA patients have been described. In addition to the secretion of matrix degrading proteinases, rheumatoid synovial fibroblasts can secrete factors like vascular endothelial growth factor (VEGF)[29] and transforming growth factor (TGF)-β[30,31], that promote the formation of new bloodvessels in the synovium,[32,33] and IL-16, which has been demonstrated to have a chemoattractive effect on T helper cells.[34] Moreover, rheumatoid synovial fibroblasts secrete osteoclast differentiation factor (ODF), which stimulates osteoclastogenesis,[35,36] suggesting that rheumatoid synovial fibroblast indirectly mediate the destruction of articular bone.

Accumulating evidence suggests that rheumatoid synovial fibroblasts have characteristics of transformed cells. The expression of several oncogenes, including *c-Myc*, *Ras*, *c-Jun* and *Jun-b*,[16,37] and the loss of contact inhibition in the presence of their key growth factor, platelet-derived growth factor[38,39] are characteristics of cells that have escaped normal cell-regulatory mechanisms. In the rheumatoid synovium, the apoptosis rate is very low[40]. The presence of mutated forms of the p53 tumor suppressor gene, which was observed in 40% of a population of RA patients,[41] may contribute to the ineffective apoptosis. However, in a German RA patient population, no specific P53 mutations were found, suggesting that suppression of apoptosis is also mediated by other mechanisms.[42] The lack of expression of the novel tumor suppressor gene, PTEN, in the synovial intima and in aggressive rheumatoid

synovial fibroblasts may also contribute to the ineffective apoptosis.[43] The most striking evidence for rheumatoid synovial fibroblast transformation is their ability to invade the articular cartilage without stimulation of inflammatory mediators. When isolated rheumatoid synovial fibroblasts are co-implanted with human articular cartilage under the renal capsule of a severe combined immunodeficient (SCID) mouse, the rheumatoid synovial fibroblasts spontaneously invade the cartilage whereas synovial fibroblasts from osteoarthritis patients or dermal fibroblasts do not.[44]

The secretion of factors mediating angiogenesis, inflammation and tissue destruction, together with the transfomed cell-like characteristics, suggest that the synovial fibroblast in RA is not just a passive responder to inflammatory stimuli, but contributes actively to the pathogenesis of RA.

4. Invasion of extracellular matrix, the role of proteinases

Invasion of the articular cartilage and bone in RA is essentially a pathological tissue remodeling process in which normal tissue is replaced by pannus tissue. The invasion of the articular cartilage by the pannus tissue in RA shares features with physiologic connective tissue remodeling such as embryonic growth and wound healing, as well as with other pathological invasive processes such as invasion and metastasis of malignant cells in cancer.[45] The degradation of the extracellular matrix (ECM) by proteinases is required for invasive cells to migrate into adjacent tissues. The proteinases that are involved in physiological tissue remodeling (embryonal growth, wound healing) are often also involved in pathologic tissue remodeling (cancer invasion and metastasis).[46-48] Since the ECM is built up by a variety of molecules, a complex array of proteinases is required for its degradation (Figure 5). Cartilage, for instance, is built up by collagens, proteoglycans, glycoproteins, and other molecules. Due to its unique composition, helical collagen, is resistant to most proteinases, except for specific collagenases that make a single cleavage in the molecule. This causes the triple helix to denature and makes it susceptible to further degradation by gelatinases or broad-spectrum proteinases.

The ECM-degrading enzymes produced by most invasive cells can be divided into four catalytic types: 1) serine proteinases, 2) metalloproteinases, 3) cysteine proteinases and 4) aspartate proteinases (table 1, 2, 3). This classification is based on the catalytic mechanism. Serine proteinases require a serine residue, metalloproteinases require a divalent cation (usually zinc, but sometimes cobalt or manganese), cysteine proteinases require a cysteine residue, and aspartate proteinases require two aspartate residues in their catalytic domain. In addition to the proteinases mentioned above, endo- and exoglycosidases contribute to ECM degradation by selectively hydrolyzing glycosaminoglycans and aminosugar moieties of proteoglycans.[45,49] The aspartate proteinases and cysteine proteinases, including the cathepsins, function at low pH and are involved mainly in intracellular proteolysis within

lysosomes. The metalloproteinases and the serine proteinases, including the plasminogen activators and plasmin, are active at neutral pH and are mainly responsible for extracellular proteolysis.

In tumor invasion, representatives of all classes are implicated, but most evidence is reported of the involvement of the plasminogen activation system and the matrix metalloproteinases (MMPs).[46,49-61]

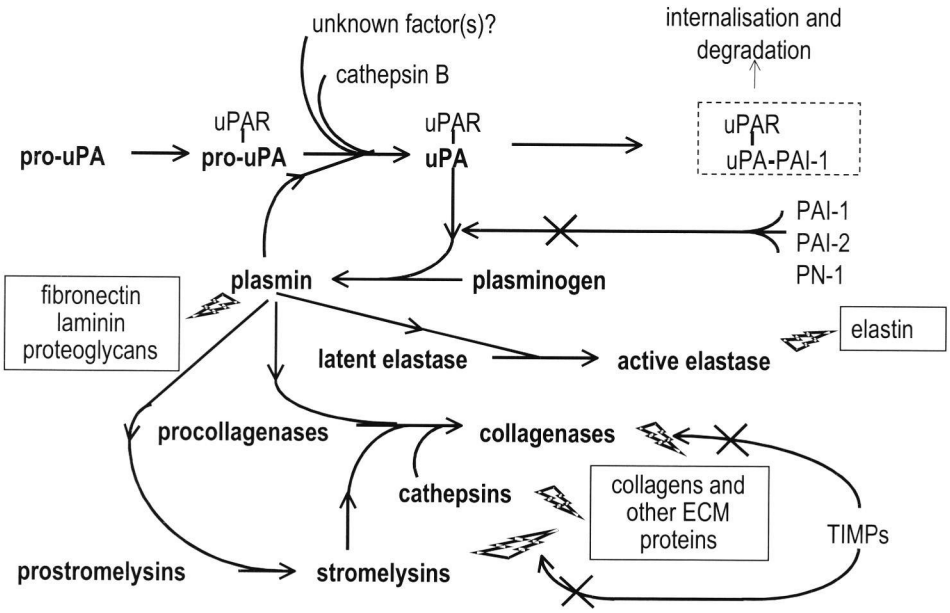

Figure 5. The interaction of serine proteinases, MMPs, cysteine proteinases and their inhibitors in extracellular matrix (ECM) turnover. *Proteolytic enzymes interact with each other with respect to activation of pro-enzymes and degradation of ECM molecules. The substrates of the different proteinases are given in the squares. The ability to degrade substrates is indicated by* ↯. *Inhibition of proteolytic activity is indicated by* —⊗→ .

Serine protease	Source	ECM substrate	Activated by
Neutrophil elastase	PMN leukocytes	Collagen type I, III, IV*,VIII, IX, X, XI; fibronectin; laminin; gelatin; elastin; proteoglycans[§]	
Cathepsin G	PMN leukocytes	Collagen type I*, II*, III, IV, VI; proteoglycans[§,¥]; gelatin; elastin; fibronectin	
Proteinase 3	PMN leukocytes	Fibronectin; laminin; vitronectin; collagen IV; elastin	
Plasmin	Plasma	Gelatin; fibronectin; laminin; proteoglycans[§]	uPA; tPA
Plasma kallikrein	Plasma		Coagulation factor XII
Tissue kallekrein	Glandular tissues		
tPA	Endothelial cells; chondrocytes		
uPA	Fibroblasts; chondrocytes		Plasmin; kallikrein, factor XIIa; cathepsin B; Cathepsin G; tryptase
Tryptase	Mast cells	Collagen IV, VI; fibronectin	
Chymase	Mast cells	Proteoglycans[§]; collagen IV, VI	
Granzyme A	Natural killer cells; Cytotoxic T-cells; granular lymphocytes	Collagen IV; fibronectin; proteoglycans	
Granzyme B	Natural killer cells; Cytotoxic T-cells; granular lymphocytes	Aggrecan	

Table 1. Serine proteases involved in ECM turnover. ** telopeptide only; [§] core protein; [¥] link protein.*

(Next page)

Table 2. Matrix metalloproteinases involved in ECM turnover. *Note that several proteinases, including plasmin and certain MMPs are also involved in the activation of MMPs. * telopeptide only; [§] core protein; [¥] link protein; [a] reviewed by Ravanti et al.*[62]

MMP group	Source	ECM substrate	Activated by
Collagenases			
Collagenase 1 (MMP-1)	Fibroblasts, chondrocytes	Collagen I, II, III, VII, VIII, X; proteoglycans;	MMP-3, -10; plasmin; kallekrein; chymase
Collagenase 2 (MMP-8)	Fibroblasts; endothelial cells	Collagen I, II, III; proteoglycans[§];	MMP-3, -10; plasmin
Neutrophil collagenase (MMP-8)	PMN Leukocytes	Collagen I, II, III;	MMP-3, -10; plasmin
Collagenase 3 (MMP-13)	Espressed only during rapid physiologic and pathologic tissue turnover	Collagen I, II, III; IV, IX, X, XIV; gelatin; fibronectin; laminin; large tenascin C; proteoglycans; fibrilline; osteonectin	MMP-2, -3, -10, -14, -15; plasmin
Gelatinases			
Gelatinase A (MMP-2)	Fibroblasts, chondrocytes	Proteoglycans[§,¥]; collagen IV, V, VII, XI; fibronectin; elastin; laminin, entactin; tenascin; fibrillin; osteonectin	MMP-1, -7, -13, -14, -15, -16, -17, -24, 25, tryptase?
Gelatinase B (MMP-9)	PMN Leukocytes; macrophages; fibroblasts; chondrocytes; ostoclasts	Proteoglycans[§,¥]; collagen IV, V, VII, XI; XIV; elastin; fibronectin; elastin; laminin; entactin; fibrillin; osteonectin	MMP-2, -3, -13, plasmin
Stromelysins			
Stromelysin 1 (MMP-3)	Fibroblasts; chondrocytes; keratinocytes	Proteoglycans[§,¥]; collagen I*, II*, III (weak), IV, V, VII; IX, X, XI*, gelatin; fibronectin, elastin, laminin	Plasmin; kallikrein; chymase; tryptase
Stromelysin 2 (MMP-10)	Keratinocytes	Proteoglycans[§,¥]; collagen IV, V, VII; IX, X; gelatin; fibronectin, elastin, laminin	
Stromelysin 3 (MMP-11)	Fibroblasts		Furin
Matrilysin (MMP-7)	Macrophages; fibroblasts	Proteoglycans[§,¥]; collagen IV; elastin; fibronectin; laminin; tenascin; entactin	MMP-3, -10; plasmin
Membrane type (MT) MMPs			
MT1- MMP (MMP-14)	Fibroblasts; endothelial cells	Collagen I, II, III, gelatin; vitronectin; proteoglycans; tenascin; fibrillin;	Plasmin; furin
MT2-MMP (MMP-15)	Several human normal and tumour tissues[a]	Fibronectin; laminin; proteoglycans; tenascin	?
MT3-MMP (MMP-16)	Several human normal tissues[a]	Collagen III; fibronectin; gelatin; casein; proteoglycans; vitronectin; laminin	?
MT4-MMP (MMP-17)	Leukocytes; several human normal tissues[a]	Gelatin	?
MT5-MMP (MMP-24)	Several human normal tissues[a]	?	?
MT6-MMP (MMP-25)	Leukocytes; several human normal tissues[a]	?	?
Other MMPs			
Metalloelastase (MMP12)	Macrophages	Proteglycans; collagen IV; gelatin; fibronectin; laminin; vitronectin; elastin; fibrillin	
MMP-19	Edothelial cells; fibroblasts; skin	Gelatin	Trypsin
Enamelysin (MMP-20)	Dental tissue	Amelogenin	?
MMP-23	Ovary; testis; prostate	?	?
MMP-26	Uterus	Gelatin; α_2M	?

Proteinase	Source	Substrate	Active at
Cysteine proteinases			
Cathepsin B	Lysosomes; secreted by macrophages, osteoclasts	Aggrecan[§,¥]; collagen I*	Neutral pH
Cathepsin L	Lysosomes; secreted by macrophages, osteoclasts	Aggrecan[§]; collagen I*, IX, XI; elastin	Low pH
Cathepsin S	Lysosomes; secreted by macrophages, osteoclasts	Proteoglycans; collagen I*, IX, XI; elastin	Neutral pH
Calpain	Cytosol	Aggrecan[§];	
Aspartic proteinases			
Cathepsin D	Lysosomes of most cells; secreted by macrophages, fibroblasts	Aggrecan	Low pH

Table 3. Cystein and aspartic proteinases involved in ECM turnover.
** telopeptide only; [§] core protein; [¥] link protein.*

5. The plasminogen activator (PA) system

5.1. The PA-system: proteinases and inhibitors

Plasmin is an important enzyme in physiological proteolytic processes including fibrin clot lysis in the circulation[63] and in the turnover of the ECM.[64] Plasmin has a broad spectrum of substrates including fibrin, proteoglycans,[65] gelatins, fibronectin, and laminin.[66,67] Moreover it has the capacity to activate other enzymes such as the MMPs and one of its own activators, urokinase-type plasminogen activator (uPA)[68-70] (Figure 5; table 1). If a broad-spectrum proteinase like plasmin were to circulate in its active form, proteolytic degradation would occur throughout the body. Therefore, plasmin is tightly regulated: first, it is secreted in its pro-form plasminogen; second, the expression of plasminogen activators is regulated by several factors including cytokines, adhesion molecules, and cell-matrix interaction; third, free plasmin is immediately inhibited by an abundance of inhibitors.[71,72] Plasminogen is a 90 kD glycoprotein and its concentration in serum and interstitial fluid is approximately 100-200 µg/ml. The liver is the principal site of synthesis, but granulocytes and the kidney can also synthesise plasminogen.[73] The abundance in the circulation and the extracellular compartments allows plasminogen to be readily available anywhere in the body where plasmin activity is required. Two plasminogen activators have been described: uPA and tissue-type plasminogen activator (tPA). Both plasminogen activators are secreted as single chain proteins. Single chain uPA has little or no activity. It is activated by cleavage of a single peptide bond resulting in the formation of two chains that remain linked by disulfide bonds.

This cleavage takes place in the extracellular compartment and can be mediated by plasmin, kallikrein, factor XIIa or cathepsin.[74-77] Unlike uPA, both single chain and two chain tPA have proteolytic activity. In contrast to uPA, the activity of tPA is strongly enhanced by binding to fibrin(ogen).[78] On the other hand, uPA binds to the uPA receptor (uPAR) at the cell membrane of a variety of cells, converting cell membrane-bound plasminogen to plasmin.[79-83] These distinct properties have led to the generally accepted view that tPA is the principal activator of fibrin dissolution, whereas uPA is implicated in pericellular ECM degradation. In response to adhesion molecules such as vitronectin, uPAR accumulates at the invasive front of the cell membrane.[84] Plasminogen binds to the cell membrane in close proximity to the uPA receptor (uPAR), allowing efficient plasminogen activation by uPAR-bound uPA at sites of ECM invasion.[85-87]

In the circulation and extracellular fluids, plasmin is mainly inhibited by α_2-antiplasmin, whereas in the ECM plasminogen activator inhibitor (PAI)-1 and PAI-2 are responsible for plasmin inhibion. However, when plasmin is bound to the cell surface, it is less susceptible for its inhibitors.[88] As such, active cell surface-bound plasmin can mediate proteolysis of the ECM in the direct environment of the cells.

5.2. The PA-system and joint destruction in RA

Fibrin and the fibrinoloytic system have been implicated in RA since the 1960s. In the rheumatoid joint, intra-articular fibrin depositions are a common feature and it was observed that the amount of intra-articular fibrin was related to different grades of joint inflammation.[89] This led to the idea that unresolved fibrin depositions may enhance the persisting inflammation and may contribute to the development of joint damage. Furthermore, increased expression of components of the PA-system, in particular uPA, uPAR and PAI-1, was observed in the rheumatoid synovium as compared with osteoarthritis or non-arthritic synovium.[90,91] The localisation of uPA, uPAR and PAI-1 at the hyperplastic synovial lining, the invasive front of the pannus tissue, suggest that these enzymes may play a role in the destruction of the articular cartilage and bone. When compared with non-arthritic synovial fibroblasts, increased production of uPA, the uPA-receptor,[92] and proteins involved in plasminogen binding was observed in rheumatoid synovial fibroblasts.[93] Increased levels of components of the PA-system were also observed in the synovial fluid[94,95] and in the circulation.[96-98] In contrast, tPA levels in the synovial fluid or plasma are decreased in RA patients.[95] Levels of synovial fluid and plasma of uPA,[98] soluble uPAR,[96] and PAI-1[99] showed a relationship with parameters of disease activity or joint damage, suggesting that the PA-system may indeed be involved in the joint pathology in RA. Furthermore, *in vitro* evidence showed that plasmin can mediate proteoglycan degradation[65] and also, in co-operation with IL-1, the degradation of collagen.[100-102] *In vivo* results from studies investigating the effects of plasmin inhibition in experimental and rheumatoid arthritis contribute to the concept of a role

of the PA-system in joint destruction. Treatment with the plasmin inhibitor tranexamic acid showed a significant reduction of urinary excretion rates of hydroxylsylpyridinoline (HP) and lysylpyridinoline (LP), collagen cross-links that are excreted in the urine upon collagen degradation, in experimental arthritis in rats as compared to placebo,[103] as well as in RA patients in an open uncontroled pilot study[103,103] (see also paragraph 8.1).

6. Matrix metalloproteinases (MMPs)

6.1. MMPs and inhibitors

The MMPs are a family of zinc-dependent proteolytic enzymes that are divided into the following subgroups: collagenases, gelatinases, stromelysins, and membrane-type (MT) MMPs (table 2). All MMPs have a similar domain structure, with a "pre" region to target for secretion, a "pro" region to maintain latency, and an active catalytic region that contains the zinc-binding active site. Most MMPs have additional domains, such as a hemopexin region or a fibronectin-like region. These additional regions are important in substrate recognition and in inhibitor binding.[104] Unlike the other MMPs, the MT-MMPs are not secreted, but remain attached to the cell surface by a transmembrane domain.[105] MMPs have the ability to degrade almost all proteins of the extracellular matrix and are believed to be key enzymes in tissue turnover processes such as embryonic growth, wound healing, tumor invasion and arthritis. Being very potent enzymes, MMPs need to be carefully controled, in a similar manner to plasmin. This takes place on the levels of synthesis, secretion, activation, and inhibition. Synthesis and secretion are regulated by factors like cytokines, growth factors, oncogenes, or hormones.[106] Just like plasmin, MMPs are secreted as inactive pro-enzymes. After secretion, the pro-MMPs are activated at specific sites by the removal of the pro-peptide by proteinases, including plasmin and other MMPs.[104,107] Free active MMPs are inhibited immediately by an abundance of circulating inhibitors, mainly α_2-macroglobuline, and tissue inhibitors of metalloproteinases (TIMPs).[56]

Four subtypes of TIMPs have been described,[108] which have overlapping as well as distinct properties. TIMP-1, TIMP-2, and TIMP-4 are secreted as soluble factors, while TIMP-3 is associated with the extracellular matrix. TIMP-2 and TIMP-3, but not TIMP-1, are effective inhibitors of the MT-MMPs. Moreover, in contrast to the other TIMPs, TIMP-3 is capable of inhibiting the shedding of cell membrane-anchored proteins such as TNF-receptor,[109] IL-6 receptor,[110] syndecan ectodomains,[111] and of inhibiting TNF-α converting enzyme (TACE).[112] Another intriguing property of TIMP-3, in contrast to the other TIMPs, is its ability to enhance apoptosis in endothelial cells, smooth muscle cells, and malignant cells.[113-115]

6.2. MMPs and joint destruction in RA

A large number of studies indicate the involvement of MMPs in joint destruction in RA. In rheumatoid synovial tissue, increased amounts of MMPs are expressed.[116-121] MMP levels in the synovium and serum are correlated with disease activity[122] and radiographic damage,[123,124] suggesting a role for MMPs in the destructive process in RA. MMP expression is stimulated by proinflammatory cytokines including TNF-α and IL-1-β,[125] which are produced by the synovial macrophages in the rheumatoid joint. The presence of TIMP-1, TIMP-2 and TIMP-3 has been shown in RA synovial tissue.[116,126] Despite an increased expression of TIMPs in RA,[116,127] proteolytic degradation of the articular cartilage still occurs, suggesting that there is still an imbalance of MMP and TIMP activity in favor of the MMPs.

7. Other proteases and joint destruction in RA

7.1. Cathepsins

Besides the plasminogen activation system and the matrix metalloproteinases, there is also evidence of the involvement of cathepsins in joint destruction in RA. Cysteine proteases are capable of degrading several cartilage matrix components, including proteoglycans, collagens type I, II, IX, and XI, and basement membrane components.[128] Moreover, cathepsins, in particular cathepsin B, are potent activators of pro-uPA.[76,77,129] Cathepsin B is present in most, but not all, synovial tissues of patients with advanced RA and is associated with the synovial fibroblast at the invasive front of the pannus tissue.[130] Abundant expression of cathepsin L has also been observed at sites of cartilage invasion,[131] suggesting a role in cartilage destruction. Cathepsin K is a mediator of osteoclast-mediated bone resorption and has also been found in the synovium of RA patients at sites of bone destruction. Cathepsin K was not only found in osteoclast precursors, but also in synovial fibroblasts at cartilage-pannus in the surrounding of lymphocyte infiltrates, suggesting that it also may contribute to joint destruction by facilitating the movement of mononuclear cells through the ECM.[132]

7.2. Granzymes

Granzymes are soluble cytolytic proteinases able to induce apoptosis in target cells in the presence of perforin.[133] Granzyme A is a proteinase with trypsin-like activity; granzyme B specifically cleaves behind aspartic acid residues. If the lysosome-like granules of activated cytotoxic T-lymphocytes and natural killer cells are released from the cells, granzymes may have extracellular effects.[134,135] Granzyme A can stimulate the production of IL-6 and IL-8 by fibroblasts and epithelial cells as well as that of IL-6, IL-8 and TNF-α by monocytes.[136,137]

Furthermore, these enzymes may be involved in the remodeling of the extracellular matrix, as illustrated by the capacity of granzyme A to degrade basement membrane type IV collagen.[138-142] Several observations suggest the involvement of granzymes in joint inflammation and destruction in RA patients. In the synovial fluid of patients with RA, lymphocytes have been shown to express granzyme A messenger RNA.[143,144] Recently, increased concentrations of soluble granzyme B were found in synovial fluid of RA patients when compared with patients suffering from osteoarthritis or reactive arthritis.[145] The levels of soluble granzyme B were significantly higher in synovial fluid than in corresponding plasma samples indicating local production within the inflamed joint. These observations in synovial fluid are in line with those in rheumatoid synovial tissue where the presence of granzyme A[146-148] and granzyme B[147,148] has been reported. The number of granzyme B positive cells, mainly natural killer cells, was found to be specifically elevated in patients with RA and the degree of expression correlated positively with parameters of arthritis activity[148]. Whether granzymes are actively involved in articular cartilage destruction in RA remains to be elucidated and is studied in Chapter 3 of this thesis.

8. Treatment of RA: prevention of joint destruction

Therapeutic strategies for RA patients have in general aimed at a reduction of inflammation. The traditional therapeutic strategy starting with non-steroidal anti-inflammatory drugs (NSAIDs), followed by the introduction of disease-modifying anti-rheumatic drugs (DMARDs), did not proof to be satisfactory on the long term, particularly with respect to the progression of joint destruction. Treatment with a combination of DMARDs in an early stage of the disease has improved the outcome of RA. It has been shown that the combination of sulfasalazine, methotrexate and predisolone in patients with early RA reduced the progression joint destruction after 80 weeks of follow-up.[149] Novel therapies with inhibitors of proinflammatory cytokines also have shown promising results with respect to a reduction of the progression of joint destruction, [150,151] but the results of these therapies on the long term need to be awaited.

Novel therapeutic strategies have improved the treatment of RA, but the prevention of destruction of the joints is still a challenge. Even though persistent inflammation is related to the progression of joint destruction,[152] inhibition of inflammation alone may not be sufficient to block the progression of joint destruction in RA patients. It has been shown that in patients with inactive RA, the urinary excretion rates of collagen cross-links were still significantly higher than those in healthy controls.[153] This suggests that joint destruction may continue in the absence of synovial inflammation and that additional treatment aimed at the direct inhibition of the destructive process may be necessary for optimal inhibition of joint destruction in RA patients.

8.1. Proteinase inhibitors as therapeutic strategy in RA

Targeting proteinases, which are directly responsible for tissue destruction in RA, may be a strategy to inhibit joint destruction in RA. A few studies have investigated the effect of proteinase inhibitors in RA. The effect of plasmin inhibition on joint destruction in RA patients was studied in an open, uncontroled pilot study. As indicators for articular cartilage and bone degradation urinary excretion rates of collagen cross-links HP and LP were measured (see paragraph 9.1). During treatment with tranexamic acid the excretion rates of HP and LP decreased and returned to baseline after cessation of treatment, suggesting that the observed effect was due to tranexamic acid treatment.[103] In chapter 6 of this thesis, a double-blind placebo-controlled pilot study is described investigating the effects of tranexamic acid on HP and LP excretion rates and on parameters of disease activity in patients with RA.

The effects of MMP inhibitors, in particular tetracyclines, have been studied more extensively in RA patients. Tetracyclines are potent MMP inhibitors, a characteristic that is unrelated to their antibiotic function.[154-158] In patients with joint disease, a significant reduction of MMP activity within the joints was demonstrated after oral administration of minocycline[156] or doxycycline.[159] Nevertheless, double-blind placebo-controlled studies investigating the effects of minocycline in RA revealed that minocycline has no effect on the progression of joint damage, but instead has effects on parameters of inflammation.[160-163] The effect of low-dose doxycycline (20 mg twice a day) on urinary HP excretion rates was investigated in an open, uncontroled study. In this study it was observed that in patients with mild RA, HP excretion rates were significantly reduced during doxycycline treatment.[164] This observation combined with *in vitro* and *in vivo* evidence of a potential beneficial effect of doxycycline on articular cartilage and bone destruction, led to the double-blind placebo-controled study investigating the effects of doxycycline in RA patients, which is described in Chapter 7 of this thesis.

Synthetic MMP inhibitors have been developed by pharmaceutical companies for their potential therapeutic benefit in MMP-related diseases, such as metastatic malignancies and RA. Unfortunately, the most common side effects include arthralgias, polyarthritis and stiffening of the joints,[165,166] which make these drugs unsuitable to use in RA patients. The synthetic MMP inhibitors available are not selective, but inhibit MMPs in general. Therefore, they are likely to cause side effects in connective tissues that undergo physiologic extracellular matrix turnover.

8.2. Gene therapy

Gene therapy offers the possibility of targeting proteinases that are implicated in the development of joint destruction in RA by the delivery of genes encoding specific inhibitors at the site of destruction. In diseases with localized pathology, such as localized tumors or

arthritis, the direct delivery of genes into the tumor or synovium has the benefit of localizing the therapeutic agent in high concentrations to the site of interest and limiting systemic side effects. Moreover, the therapeutic genes inserted have the potential to be expressed for a long period of time in stable concentrations, whereas the half-life of proteins that are injected parenterally varies from minutes to days. For chronic diseases such as RA this may be an important benefit.

For the efficient delivery of genes into cells, the DNA needs to be packaged in a gene delivery device, a so-called vector, in such a way that it enters a cell easily. Viruses are natural gene delivery devices, as they insert their DNA directly into a cell upon infection in order to use the cell for replication. By modulating the virus, vectors can be made that can be used for gene delivery. Molecular biology techniques have created the possibility of deleting unwanted parts of the virus DNA, such as the genes necessary for replication, and insert genes encoding a therapeutic or reporter gene (Figure 6).

Thus far, most experience has been gained with adenoviruses and retroviruses.[167] Retroviral vectors have the property that the gene is incorporated into the genomic DNA, allowing the transgene to be passed on during cell division. Only dividing cells are sensitive to infection, which limits its use *in vivo* if non-dividing cells are a target. Adenoviral vectors have a high transduction efficiency.[168] and the ability to infect both dividing and non-dividing cells[169] The inserted gene is not incorporated into the genome and the transgene and during proliferation the transgene is passed on to only one daughter cell. In tissues with high proliferation rates, the percentage of transduced cells rapidly decreases.

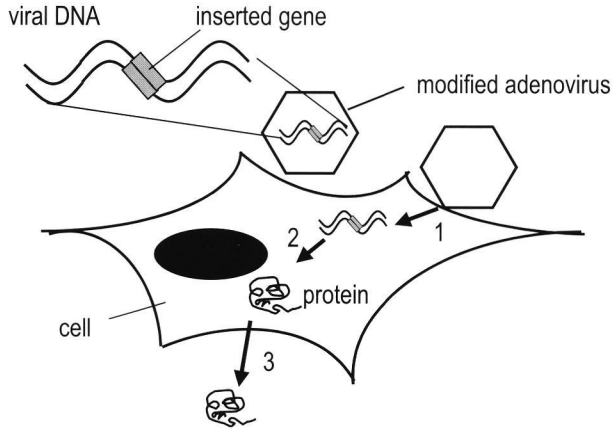

Figure 6. Schematic representation of adenoviral gene transfer. *A modified adenovirus, containing the gene of interest, infects a cell (1). The promoter accompanying the gene of interest results in transcription of the DNA and translation into a protein (2). The protein is subsequently secreted by the cell (3).*

The immune response that is triggered by infected cells is another cause of loss of transgene expression. This is particularly the case for adenoviral vectors, which are derived from common cold viruses.[170] Nevertheless, the currently available vectors are suitable for preclinical studies investigating the feasibility of such an approach and investigating different targets for gene therapy.

Several *in vitro* and *in vivo* studies in the field of arthritis have focused on targeting proinflammatory cytokines by transfer of genes encoding antibodies to proinflammatory cytokines or genes encoding anti-inflammatory cytokines.[171-174] Other approaches are the reduction of the hyperplastic rheumatoid synovial tissue by the induction of apoptosis[175-177] or by inserting a 'suicide' gene, for instance thymidine kinase that causes cell death in dividing cells when the cells are subjected to ganciclovir.[168] An approach that is currently gaining interest in arthritic disorders is the targeting of proteinases involved in joint destruction. In the field of cancer and cardiovascular disease some experience has been gained with the transfer of genes encoding TIMPs,[114,115] revealing that cell migration processes can be inhibited by gene transfer of TIMPs. In chapter 5 and 6 the possibilities of proteinase-targeted gene transfer in rheumatoid arthritis was explored.

9. Molecular markers of joint destruction

When RA patients are treated with a (potential) joint-sparing drug, it is useful to measure the effectiveness of such therapy with simple laboratory tests that reflect the destruction of the articular tissues. Molecules of fragments of molecules, such as collagens, proteoglycans, or other macromolecules that are released in the synovial fluid and subsequently in the circulation upon degradation of articular cartilage and bone can be used as molecular markers of joint destruction in RA. Examples of collagen degradation markers are collagen cross-links[178] and C- or N-terminal telopeptides of collagen molecules.[179,180] Examples of markers of proteoglycan degradation are chondroitin sulfate, keratan sulfate, or hyaluronic acid[181]. Cartilage oligomeric protein is an example of another cartilage macromolecule that can be used as marker of cartilage degradation.[182] Because the collagen cross-links HP and LP have been used as molecular markers for joint destruction in Chapter 5 and 7 of this thesis, they are discussed below.

9.1. Collagen cross-links

The pyridinolines HP and LP form cross-links between three collagen molecules by connecting the triple helices of one collagen with the telopeptides of two collagen molecules (Figure 3B). HP is found in various tissues including cartilage, bone, and ligaments, but the

highest concentrations of HP are found in articular cartilage.[183] LP is mainly found in bone and dentine.[183] HP and LP are released into the circulation upon the degradation of collagen fibrils and excreted unchanged in the urine. It has been observed that in conditions with an increased state of cartilage and/or bone degradation, such as osteoporosis and RA, levels of urinary HP and LP are increased (reviewed in Seibel et al.).[184] It has been shown that pyridinoline excretion levels correlate well with disease activity parameters such as CRP.[178,185,186] Moreover, pyridinoline excretion levels in patients with erosive RA are significantly higher compare with those in patients with only a few or no erosions,[178] suggesting that urinary pyridinoline excretion rates may be useful as molecular markers of the process of joint destruction in RA patients.

10. Outline of this thesis

- ✓ To investigate whether the rheumatoid synovial fibroblast is influenced by cell-cell interactions, the effects of interaction of chondrocytes with RA synovial fibroblasts on the invasive potential of the RA synovial fibroblasts are described in **Chapter 2**.
- ✓ To further investigate the involvement of proteinases in joint destruction in RA, the role of granzym B in cartilage destruction in RA is explored in **Chapter 3**.
- ✓ The role of the plasminogen activator system in cartilage destruction in RA and a strategy to inhibit plasmin-mediated cartilage destruction by gene transfer are described in **Chapter 4**.
- ✓ To investigate whether plasmin inhibition may be a way to inhibit the destruction of the joints in RA patients, the effects of tranexamic acid, an inhibitor of plasminogen activation, on parameters of joint destruction and disease activity in patients with RA were evaluated. This is described in **Chapter 5**.
- ✓ The role of MMPs in cartilage invasion by RA synovial fibroblasts and the effects of gene transfer with TIMPs are described in **Chapter 6**.
- ✓ To investigate whether inhibition of MMPs may be a way to inhibit the destruction of the joints in patients with RA, the effects of doxycycline, an antibiotic with MMP-inhibitory activity, on parameters of joint destruction and disease activity in patients with RA were evaluated. This is described in **Chapter 7**.
- ✓ In **Chapter 8** the findings in this thesis are summarized and discussed.

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2

MODULATION OF FIBROBLAST-MEDIATED CARTILAGE DEGRADATION BY ARTICULAR CHONDROCYTES IN RHEUMATOID ARTHRITIS

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Arthritis & Rheumatism 2000;43:2531-2536

Summary

Destruction of the articular cartilage in rheumatoid arthritis (RA) is caused by the invasion of synovial cells into the cartilage. Here, the role of chondrocytes and factors released from chondrocytes in cartilage destruction by rheumatoid arthritis synovial fibroblasts was investigated.

Synovial fibroblasts from two RA patients were implanted into severe combined immunodeficient (SCID) mice, together with fresh articular cartilage or with cartilage stored for 24 hours at 4°C or 37°C. The invasion of the same synovial fibroblasts into the fresh and stored cartilage was compared histologically using a semiquantitative scoring system. In addition, we investigated whether protein synthesis in chondrocytes affects the invasion of rheumatoid synovial fibroblasts *in vitro*. A 3-dimensional cartilage-like matrix formed by cultured chondrocytes was labeled with ^{35}S. After formation of the cartilage-like matrix, protein synthesis was blocked with cycloheximide. The invasion of rheumatoid synovial fibroblasts from 6 patients into cycloheximide-treated and untreated matrix was assessed by measuring the released radioactivity in coculture with or without interleukin-1-β and tumor necrosis factor-α (TNF-α).

The SCID mouse experiments showed a clear invasion of the rheumatoid synovial fibroblasts into the cartilage (overall mean score 3.2) but revealed significant differences when the invasion of the same rheumatoid synovial fibroblasts into fresh and stored cartilage was compared. RA synovial fibroblasts that were implanted with fresh articular cartilage showed a significantly higher invasiveness than those implanted with pieces of cartilage that had been stored for 24 h (overall mean score 2.3). Storage at 37°C and 4°C resulted in the same reduction of invasion (35% and 37% respectively). In the *in vitro* experiments, rheumatoid synovial fibroblasts rapidly destroyed the cartilage-like matrix. Blocking of chondrocyte protein biosynthesis significantly decreased the invasion of the rheumatoid synovial fibroblasts as shown by a decreased release of radioactivity. Addition of IL-1-β, but not of TNF-α, to the cocultures partially restored the invasiveness of rheumatoid synovial fibroblasts.

These data underline the value of the SCID mouse in vivo model of rheumatoid cartilage destruction and demonstrate that chondrocytes contribute significantly to the degradation of cartilage by releasing factors that stimulate rheumatoid synovial fibroblasts. Among those, IL-1-β-mediated mechanisms might be of particular importance.

Introduction

Rheumatoid arthritis (RA) is a chronic crippling disorder in which the progressive destruction of joints represents the most prominent feature. The disease is characterized by hyperplasia and chronic inflammation of the synovial membranes that, in the course of disease, invade deeply the articular cartilage and bone. It is well established that activated rheumatoid synovial fibroblasts in the lining layer of the hyperplastic synovium contribute significantly to this process.[1,2] These cells display an altered morphology and are characterized by a complex up-regulation of intracellular signaling pathways that ultimately results in the expression of adhesion molecules and matrix-degrading enzymes. Although there is evidence that the activation is an intrinsic feature of these cells, several studies have demonstrated that pro-inflammatory cytokines enhance the activation of rheumaotid synovial fibroblasts.[3,4] Among them, the tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1-β) have been implicated most convincingly in the stimulation of activated rheumatoid synovial fibroblasts. IL-1-β in particular appears to be critically involved in the destructive process by up-regulating matrix metalloproteinases and cathepsins.[5] Since macrophages constitute the major source of such proinflammatory cytokines in the inflamed synovial tissue, these cells modulate the invasion of rheumatoid synovial fibroblasts through the release of cytokines such as IL-1.[6]

Although there is evidence that cartilage-associated molecules such as fibronectin enhance the aggressive growth of the rheumatoid synovium,[7] the question as to whether chondrocytes of the articular cartilage may contribute specifically to the stimulation of rheumatoid synovial fibroblasts and thus to the fibroblast-mediated degradation of their pericellular matrix has not yet been addressed. Rather, it has been anticipated that rheumatoid synovial fibroblasts, in concerted action with synovial macrophages and, potentially, T-cells invade the cartilage in a one way manner. The perichondrocytic degradation that can be frequently observed in rheumatoid cartilage has been attributed mainly to the stimulatory effect of macrophage-derived cytokines such as IL-1-β that stimulate not only rheumatoid synovial fibroblasts, but also chondrocytes. Consequently, several *in vitro* systems have been established that use pieces of articular cartilage to study the invasive behavior of rheumatoid synovial fibroblasts.[6-10]

We report herein that the invasion of rheumatoid synovial fibroblasts into articular cartilage is influenced strongly by chondrocytes, predominantly through the release of IL-1-β. Using the SCID mouse coimplantation model of RA as well as a novel *in vitro* model of cartilage degradation, we demonstrated that the invasion of rheumatoid synovial fibroblasts into articular cartilage decreases significantly when chondrocytes in the cartilaginous matrix suffer damage either from lack of nutrition or the inhibition of protein biosynthesis.

Material and Methods

Isolation of rheumatoid synovial fibroblasts.

Synovial tissue specimens were obtained from 8 patients with RA who were undergoing synovectomy at the Schulthess Clinic (Zürich, Switzerland). Rheumatoid synovial tissues were cut into small pieces and digested enzymaticaly (overnight in the presence of Dispase I). The released cells were grown in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS) over 4-5 passages. The culture supernatant was tested by ELISA for infection with *Mycoplasma* species. Rheumatoid synovial fibroblasts from 2 RA-patients were used in the SCID mouse experiments while rheumatoid synovial fibroblasts from 6 patients were used in the *in vitro* model of cartilage degradation. In the SCID mouse experiments, normal synovial fibroblasts from 1 subject who had no history of joint disease was used as control.

Articular cartilage

Normal human articular cartilage was obtained from patients undergoing joint replacement surgery for sever trauma at the Department of Surgery, University Hospital (Zürich, Switzerland). The cartilage was processed under sterile conditions. One part of the articular cartilage was used in the SCID mouse experiments while another portion was taken to isolate chondrocytes for the in vitro experiments.

SCID mouse co-implantation experiments

Two weeks old, female severe combined immunodeficient (SCID) mice were provided by the Charles Rivers Laboratories (Sulzfeld, Germany) and kept permanently under germ-free conditions. Implantation of RA synovial fibroblasts together with normal human cartilage was performed as described previously.[11] Briefly, after trypsinization, washing and centrifugation, 10^5 cells were resuspended in 100 µl sterile culture medium and inserted into the cavity of an inert sponge (Gelfoam, Pharmacia & Upjohn, Dübendorf, Germany) together with a 1-mm^3 piece of the articular cartilage. Mice were anesthetized by intraperitoneal injection of Xylocain (lidocain hydrochloride, 0.014 mg/g, Astra Pharmaceutica, Dieticon, Switzerland) and Ketalar (ketamin hydrochloride, 0.09 mg/g, Parke-Davis, Baar, Switzerland) in an isotonic solution. A 1-cm incision was made on the left flank of the animals. The left kidney was exteriorized, a small incision made, and an implant was placed under the renal capsule. The peritoneal layer and the skin were closed using 5-0 prolene suture material.

Rheumatoid synovial fibroblasts and cartilage specimens were used in two groups of experiments. In both groups, part of the rheumatoid synovial fibroblasts was coimplanted with the cartilage within 4 hours after surgery, while a second portion of cartilage was kept for 24 hours and then used with the same rheumatoid synovial fibroblasts. The differences between the groups were related to the rheumatoid synovial fibroblasts used and particularly to the cartilage storage conditions during the 24 hours period. In group 1, rheumatoid synovial

fibroblasts from 1 patient were used with cartilage specimens that had been stored at 37° C, while in group 2, rheumatoid synovial fibroblasts from a second patient were implanted with cartilage that was stored at 4°C. For each set of experiments 4 animals were used, resulting in a total of 8 animals implanted with fresh cartilage and 8 animal with stored cartilage. After 60 days, the mice were killed and the implants removed. Tissue preparation included fixation in 4% buffered formalin and paraffin embedding, according to standard procedures.

Histological evaluation

The sections were stained using conventional hematoxylin and eosin staining. Histological evaluation was performed and scored semiquantitatively for RA synovial fibroblasts invasion according previouslyly described methods.[11] The depth of invasion was assessed by estimating the of cell layers invaded and was scored as follows: 0 = no or minimal invasion, 1 = visible invasion (2-5 cell depths), 2 = invasion (6-10 cell depths), 3 = deep invasion (> 10 cell depths), and 4 = overall deep invasion that reshapes the cartilage surface.

Three dimensional cartilage-like matrix

For the *in vitro* experiments, a cartilage-like matrix was generated by growing isolated chondrocytes in 3-dimensional collagen sponges. Articular cartilage was cut into small pieces of 2-4 mm and digested enzymaticaly (Pronase for 2 hours and collagenase P for 18 hours, Calbiochem, Bad soden, Germany and Boehringer, Mannheim, Germany). Isolated chondrocytes were washed 5 times with phosphate buffered saline and twice with DMEM.

Collagen sponges (Gel foam, Pharmacia-Upjohn) were pre-treated with 100 ml Bovine extracellular matrix (B-ECM) as described elsewhere[12] and washed 3 times in 2 ml DMEM to adjust the pH and ion concentrations, and isolated chondrocytes were soaked into the pretreated collagen sponges. The sponges were cultured in 24-well plates (2 ml medium) for 1day and in 6-well plates (5 ml medium) for 20 days at 37 °C. The medium used was DMEM/F12 containing 20% FCS, 50 IU/ml penicillin-streptomycin, and 2.5 mg/ml amphotericin B (Fungizone, Gibco BRL, Eggenstein, Germany).

Radioactive labeling with sulfate.

To establish a sensitive method for measuring the degradation of the cartilage-like matrix, bovine extracelular matrix/chondrocyte sponges were labeled with ^{35}S (Amersham, Braunschweig, Germany). Briefly, 0.01 mCi of ^{35}S was added to each sponge in 1.5 ml medium. DMEM containing 20% FCS, 50 IU/ml penicillin-streptomycin and 2.5 mg/ml amphotericin B was changed daily for 10 days before coculturing the sponges with the RA synovial fibroblasts. The washing process allowed a gradient of ^{35}S incorporation over 6-7 days, minimized the risk of cell damage due to radioactivity and the return to radioactivity background level.

Co-cultures of rheumatoid synovial fibroblasrs with a cartilage-like matrix

Isolated rheumatoid synovial fibroblasts (10^5 cells) were added to bovine extracellular matrix/human chondrocyte sponges. They were cocultured in a 24-well plate for 21-28 days. DMEM/F-12 medium (1.5 ml) containing 10% FCS, 5 IU/ml penicillin-streptomycin, and 0.25 mg/ml amphotericin B was changed daily. The release of ^{35}S reflected the degradation of the cartilaginous matrix. The spontaneous release of ^{35}S was determined using sponges without RA synovial fibroblasts (Beta-matic liquid scintillation counter, Kontron, Zurich, Switzerland). Medium was centrifuged at 400 g for 10 minutes, and 100 µl supernatant was added 10 ml of Irga-Safeä Plus scintillation cocktail (Canberra Packard, Meriden, Connecticut, USA). Bovine extracellular matrix/human chondrocyte sponges were pre-treated with 1.0 mM cycloheximide (Sigma, Buchs, Switzerland) 24 hours before the addition of RA synovial fibroblasts. The sponges were washed 5 times with DMEM to eliminate cycloheximide, and rheumatoid synovial fibroblasts were added in presence of medium alone, IL-1-β, or TNF-α.

The medium in 6-well plates (5 ml) was changed weekly and the radioactivity was determined on days 7, 14 and 21. After 7 and 14 days of incubation at 37°C, sponges were transferred to wells containing fresh medium (5 ml) containing 10% FCS without other agents. The spontaneous release of ^{35}S was controlled by sponges alone. All experiments were done in triplicates using rheumatoid synovial fibroblasts from the same donor.

Statistical analysis

For statistical analysis, the means, standard deviations, and standard error of means were calculated. Differences between groups were tested for statistical difference using the Mann-Whitney U-test. Differences were considered statistically significant at $p < 0.05$.

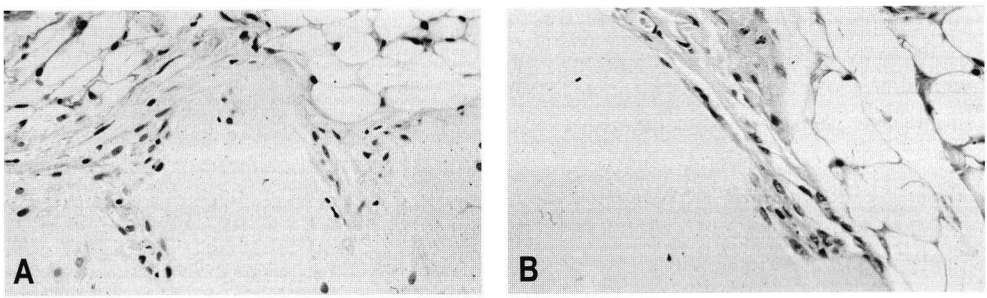

Figure 1: Invasion of RA-SF into fresh (A) and stored articular cartilage (B) in the SCID mouse co-implantation model of RA. *At implantation with the same RA-SF, invasion into the fresh cartilage (A) was greater than into stored cartilage pieces (B).*

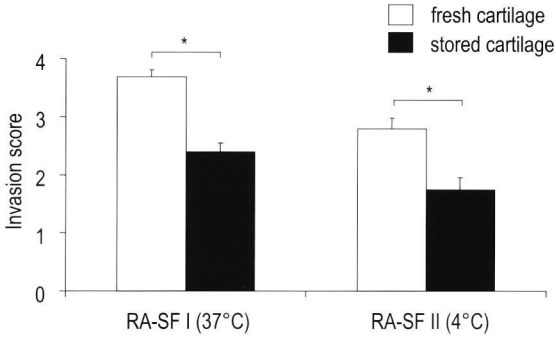

Figure 2. Comparison of the invasion scores. *Co-implantation of rheumatoid synovial fibroblasts (RA-SF) with fresh human cartilage into SCID mice resulted in invasion scores exceeding 2.5. Storage of cartilage specimens both at 4 C and 37 C significantly decreased the invasion of the rheumatoid synovial fibroblasts.*

Results

Invasiveness in the SCID mouse model

At histological evaluation, a significant invasion of the rheumatoid synovial fibroblasts into the coimplanted cartilage was seen in all samples (Figure 1). As demonstrated previously,[11] rheumatoid synovial fibroblasts growing out of the sponge attached to and deeply invaded the cartilage matrix. The mean invasion score was 3.6 for the rheumatoid synovial fibroblasts implanted with fresh cartilage in group 1 and 2.8 in group 2 (mean invasion score for all rheumatoid synovial fibroblasts 3.2). These scores corresponded to those seen in previous studies and were within the variability of invasion scores among different rheumatoid synovial fibroblasts cultures.

However, there were significant differences between the invasion of the same rheumatoid synovial fibroblasts into fresh cartilage and invasion into the pieces of cartilage that had been stored for 24 h. Rheumatoid synovial fibroblasts implanted with fresh human cartilage showed a significantly higher invasiveness than those implanted with pieces of that cartilage stored for 24 hours ($p < 0.05$, Figure 2). Interestingly, both storage at 37°C and 4°C resulted in the same reduction of invasiveness (35% and 37%, respectively) indicating that the storage conditions did not influence the reduction in invasiveness between fresh and stored cartilage.

As seen in previous studies,[11] normal synovial fibroblasts did not show a significant invasion into the cartilage (mean score 1.4), and no significant differences between invasion into fresh and stored cartilage were observed. Notably, storage of the cartilage for 24 h did not result in a structural damage at sites of invasion, as assessed by histology (Figure 1B).

Reduced cartilage destruction by blocking chondrocyte protein synthesis

Based on these data, we investigated the role of intact chondrocyte protein biosynthesis for the invasion of rheumatoid synovial fibroblasts. Specifically, we analyzed the invasiveness rheumatoid synovial fibroblasts into a radio-labeled, cartilage-like, 3-dimensional matrix produced by chondrocytes that were grown in a bovine extracellular matrix sponge.

As shown in figure 3A, addition of rheumatoid synovial fibroblasts to the sponges resulted in the release of radioactivity from the sponges over a period of 21 days due to the

degradation of the radiolabeled matrix. The release of radioactivity increased rapidly from day 7 after addition of rheumatoid synovial fibroblasts, peaked at day 14, and decreased slowly thereafter (Figure 3A). Blocking of chondrocyte protein biosynthesis by the addition of cycloheximide significantly decreased the invasion of the rheumatoid synovial fibroblasts into the cartilage-like matrix, as demonstrated by a decreased release of radioactivity (Figure3A). Released radioactivity decreased by 59 to 40%, with an average of 48% over 21 days.

Effect of cytokines on the invasivenss of RA synovial fibroblasts.

The addition of IL-1-β partially restored invasiveness of rheumatoid synovial fibroblasts. As shown in Figure 3B, a clear increase of in the released radioactivity was observed, when DMEM containing IL-1-β was added to the cycloheximide treated sponge instead of DMEM alone. The cumulative radioactivity increased by 85% and reached 84% of the radioactivity released from non-cycloheximide treated cartilage sponges. Similar to the invasion of rheumatoid synovial fibroblasts in untreated sponges, radioactivity showed a peak after 14 days and decreased slowly thereafter. Interestingly, addition of TNF-α did not restore the invasion of rheumatoid synovial fibroblasts into the cycloheximide treated cartilage-like matrix (Figure 3B).

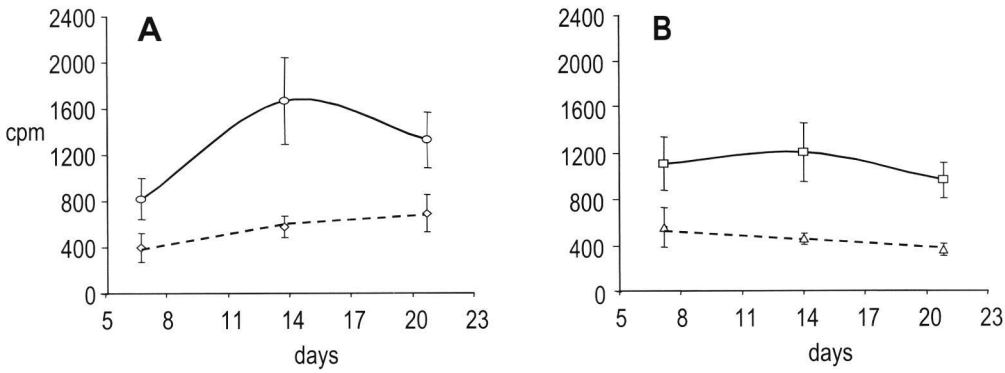

Figure 3: Degradation of a ^{35}S labeled cartilage-like matrix by rheumatoid synovial fibroblasts. The extent of matrix degradation is assessed through measuring the release of radioactivity from the matrix (full curve, A). Pre-treatment of the cartilage-like matrix with cycloheximide resulted in a significant decrease in the cumulative radioactivity released from the matrix ($p<0.05$, dashed curve, A). Addition of IL-1-β ((full curve, B) but not TNF-α (dashed curve, B) restored the invasion by rheumatoid synovial fibroblasts.

Discussion

Cartilage has been considered a major target of the hyperplastic synovium in RA. The degradation of articular cartilage not only appears to initiate joint destruction, but also to constitute a key event in the pathogenesis of RA. It has been established that fibroblast-like cells contribute significantly to this process due to their intrinsic activation as well as their stimulation by macrophage-derived cytokines, predominantly IL-1-β and TNF-α. Based on the demonstration that chondrocytes can produce proinflammatory cytokines and factors such as IL-1-β[13,14] it has been hypothesized for some time that chondrocytes may contribute to the pathogenesis of disease.[8] However, direct evidence for a significant role of chondrocytes in the cellular interplay in RA has thus far been lacking. Here, we have clearly demonstrated that cartilage is not just a passive tissue suffering destruction by activated rheumatoid synovial fibroblasts. Rather, chondrocytes appear to constitute an important part of the cellular interplay that characterizes the pathogenesis of RA.

The SCID mouse data from this study show that cartilage degradation is critically dependent on not only the properties of the rheumatoid synovial fibroblasts, but also the metabolic state of chondrocytes. This is concluded from the observation that in the SCID mouse coimplantation model, the invasion of rheumatoid synovial fibroblasts into human articular cartilage over the course of 60 days decreased significantly when the cartilage is kept outside an *in vivo* environment for 24 hours prior to implantation. Although structural damage was not observed at sites of reduced invasiveness, it is obvious that altered environmental conditions during the storage influenced the biological properties of the chondrocytes. The question of whether molecules the cartilage matrix are affected by the culture conditions deserves further investigation, but the changes most likely are due to insufficient nutrition of the cartilage causing at least partial damage to the chondrocytes.

This concept is also supported by our *in vitro* data, where blocking of the protein biosynthesis in chondrocytes resulted in a significantly decreased invasion of rheumatoid synovial fibroblasts into a newly formed cartilage-like matrix. Clearly, these data demonstrate the ability of chondrocyte derived factors to stimulate the fibroblast-mediated degradation of cartilage. Our observation that IL-1-β restores the invasiveness of rheumatoid synovial fibroblasts into the cartilage-like matrix is consistent with several studies demonstrating the potential of IL-1-β to stimulate cartilage degradation by synovial fibroblasts.[15-17] Moreover, it may be speculated that IL-1-β produced by chondrocytes may contributes to the stimulation of RA synovial fibroblasts. Alternatively, IL-1-β may stimulate articular chondrocytes to produce factors that act on synovial fibroblasts, increasing their destructive potential. Interestingly, addition of human TNF-α did not enhance the degradation of the cartilage-like matrix. Although it has been shown convincingly that high concentrations of TNF-α may enhance the destructive potential of synovial fibroblasts,[18-20] our data confirm recent observations suggesting a role for TNF-α primarily in synovial inflammation.[21]

In conclusion, the data underline the value of the SCID mouse coimplantation model as a feasible tool for investigating the destruction of cartilage by activated rheumatoid synovial fibroblasts. At the same time they point to some limitations that may be associated with *in vitro* systems that use pieces of cartilage in cell culture medium or even deep-frozen cartilage. Specifically, by not considering the interaction between rheumatoid synovial fibroblasts and chondrocytes, such models may provide data that are biased. More interestingly, our data demonstrated clearly that chondrocytes are part of the cellular interplay that characterizes the pathogenesis of RA.

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3

HUMAN GRANZYME B MEDIATES CARTILAGE PROTEOGLYCAN DEGRADATION AND IS EXPRESSED AT THE INVASE FRONT OF THE SYNOVIUM IN RHEUMATOID ARTHRITIS.

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Rheumatology 2001;40:55-61

Summary

Joint destruction in rheumatoid arthritis is mediated by proteolytic enzymes that are secreted at the pannus-cartilage junction. Granzymes are serine proteinases that are secreted by natural killer cells and cytotoxic T-cells. The elevated levels of granzymes observed in RA synovial tissue led to the investigation of the cartilage-degrading capacity of granzyme B and the presence of granzyme B-positive cells at sites of erosion in the rheumatoid synovium.
Granzyme B was added to ^{3}H proline/ ^{35}S sulfate-labeled cartilage matrices and to cartilage explants. Proteoglycan degradation was assessed by the release of ^{35}S and glycosaminoglycans into the medium and collagen degradation by the release of ^{3}H and hydroxyproline and by measuring the fraction of denatured collagen. Granzyme B expression was studied at the invasive front of the synovium by immunohistochemistry.

Granzyme B induced loss of both newly synthesized, radiolabeled proteoglycans in cartilage matrices and the resident proteoglycans of the cartilage explants. No effect on collagen degradation was found. Granzyme B-positive cells were present throughout the synovium and at the invasive front.

The presence of granzyme B-posistive cells at the invasive front of the synovium together with the ability to degrade articular proteoglycans support the view that granzyme B may contribute to joint destruction in rheumatoid arthritis.

Introduction

Proteolytic degradation of articular cartilage and bone is a characteristic feature of rheumatoid arthritis (RA). Cartilage destruction at the invasive front of the inflamed synovial tissue (pannus) has been attributed mainly to serine proteinases and matrix metalloproteinases produced by fibroblast-like synoviocytes, macrophages, chondrocytes, and polymorphonuclear cells.[1]

Recently, a set of serine proteinases, called granzymes, has been identified.[2,3] Granzymes, which include granzyme A, a proteinase with trypsin-like activity, and granzyme B, a proteinase which specifically cleaves behind aspartic acid residues, are soluble cytolytic proteinases able to induce apoptosis in target cells in the presence of perforin.[4,5] If the lysosome-like granules of activated cytotoxic lymphocytes and natural killer cells are released from the cells, granzymes may also exert extracellular effects.[6,7] Granzyme A can stimulate the production of interleukin (IL)-6 and IL-8 by fibroblasts and epithelial cells as well as that of IL-6, IL-8 and tumor necrosis factor α by monocytes.[8,9] Furthermore, these enzymes may be involved in remodeling of the extracellular matrix, as illustrated by the capacity of granzyme A to degrade basement membrane type IV collagen.[10-14] Several observations suggest a role of granzymes in joint inflammation and destruction in patients with RA. In the synovial fluid of patients with RA, lymphocytes have been shown to express granzyme A messenger RNA.[15,16] Recently, increased concentrations of soluble granzyme B were found in the synovial fluid of RA patients when compared with patients suffering from osteoarthritis or reactive arthritis.[17] The levels of soluble granzyme B were significantly higher in synovial fluid than in corresponding plasma samples, indicating local production within the inflamed joint. These observations in synovial fluid are in line with those in rheumatoid synovial tissue where the presence of granzyme A[18-20] and granzyme B[19,20] has been reported. The number of granzyme B-positive cells, mainly natural killer cells, was found to be specifically elevated in patients with RA and the degree of expression correlated positively with parameters of arthritis activity.[20]

To further investigate a potential role of granzymes in the pathophysiology of joint destruction, we assessed the capacity of granzyme B to degrade newly synthesized and resident proteoglycans and collagen in bovine articular cartilage. In addition, we investigated the presence of granzyme B-containing cells at characteristic sites of joint destruction in RA: the pannus-hard tissue junction of metacarpophalangeal (MCP) joints.

Materials and Methods

Production of radiolabelled cartilage matrix

In this study we used alginate beads to culture chondrocytes as described previously.[21] The chondrocytes were obtained from bovine metacarpophalangeal articular cartilage, isolated by

collagenase digestion, embedded in alginate, and cultured in Dulbecco's Modified Eagle's Medium, 2mM glutamic acid (DMEM Glutamax medium, Gibco Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (v/v), 50 μg/ml ascorbic acid, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% carbondioxide. After 4 weeks of culture, a cartilage matrix is produced that contains proteoglycan aggregates incorporated in a three-dimensional network of collagen type II and collagen crosslinks.[22] To radiolabel the newly formed proteoglycans and collagen fibrils in the cartilage matrix, ^{35}S-sulfate and ^{3}H-proline (Amersham International plc, Amersham, UK) was added to the culture medium. After culturing for 4 days, the culture medium was changed twice weekly with fresh medium containing 0.25 μCi/ml ^{35}S-sulfate and ^{3}H-proline. After culturing for 28 days, the alginate gel was suspended in 55 mM sodium citrate to dissolve the beads and release the synthesised matrix. The solution was centrifuged (750 g, 6 minutes) and the matrix pellet was suspended in DMEM and plated in 24 well plates. The matrix was coated for 3 days at 4°C, fixed with methanol and air-dried. Plates were stored at -20°C until use.

Degradation of newly synthesized cartilage matrix by granzyme B

Radiolabeled cartilage matrices were slowly thawed at room temperature in DMEM and incubated during 24 hours at 37°C with 1 ml natural human granzyme B at respectively 0.4 and 0.04 μg/ml in phosphate buffered saline (PBS) with 0.05% (v/v) Tween-20 (PBST) (Janssen Chemica, Geel, Belgium). Granzyme B was obtained from Evanston Hospital Corporation, Evanston, IL (lot 10.5.95, 32 units/μl, isolated from human T-cell line, YT-Indy and purified using HPLC; specific activity 16.8 units/μg). Depletion of newly synthesized proteoglycans and collagen was followed by the release of ^{35}S and ^{3}H respectively. The release of radioactivity in the medium and the remaining radioactivity in the matrix were counted by a liquid scintillation analyzer (Tri-Carb 1900 CA, Packard, Meriden, Connecticut, USA). The percentage of matrix degradation was calculated: {dpm (medium)/[dpm (medium) + dpm (matrix)]} x 100%.

Culture of bovine articular cartilage explants

Bovine metacarpophalangeal joints were acquired from a local abattoir immediately after the cows (1-2 years old) had been killed. Articular cartilage was removed aseptically from the joint, washed twice with PBS and prepared as slices with a wet weight of approximately 30 mg (dry weight approximately 10 mg consisting of 60-70% collagen type II; proteoglycans accounted for a large part of the remainder).[23] The cartilage slices were incubated in DMEM Glutamax at 37°C in a humidified atmosphere of 5% carbon dioxide. Each culture consisted of one piece of cartilage in 0.5 ml of medium.

Degradation of cartilage explants

Bovine articular cartilage explants were incubated with 1 ml of respectively 0.4 and 0.04 µg/ml human granzyme B for 15 and 90 h respectively. Proteoglycan loss was followed by measuring the release of sulfated glycosaminoglycans (GAG) in the culture medium using a commercially available assay (Blyscan, Biocolor Ltd, Belfast, U.K.), and expressed as µg/mg cartilage. Collagen degradation was assessed by measuring hydroxyproline release into the culture medium by HPLC[24] (expressed as pmol/mg cartilage) and by measurement of the fraction of denatured collagen in the cartilage explants as described previously.[25]

Immunohistochemistry

The demonstration of granzyme B-containing cells at the invasive front of the pannus tissue requires the preservation of the osteochondrosynovial transition zone for which decalcification of tissue needs to be avoided. Therefore, we embedded MCP joints in plastic before 3 µm sections were cut and incubated with the antibody specific for human granzyme B. Human metacarpophalangeal joints from 3 patients with rheumatoid arthritis were obtained at joint replacement. Slices of 3 mm were embedded in polymethacrylate.[26] The tissue was fixed by overnight incubation in acetone at a constant temperature between -15°C and -19°C. Subsequently, the tissues were impregnated by constant rotation for 6 hours at 4°C in molding cup trays (Polysciences, Warrington, Pennsylvania, USA). To this end 90 mg of benzoylperoxide containing 20-25% water (Merck, Darmstadt, Germany) was dissolved in 20 ml of 2-hydroxy-ethylmethacrylate and 20 ml of 2-hydroxy-propylmethacrylate followed by addition of 1 ml of a mixture of 6.25% (v/v) *N,N*-dimethylaniline (Merck) and 93.75% (v/v) polyethyleneglycol 400 (Fluka, Buchs, Switzerland). The polymerization mixture was stirred for 5 minutes at room temperature before addition to the tissue. Sections (3 µm) were cut on a motor-driven Reichert-Jung 2050 microtome, harvested on water containing 0.05% (v/v) ammonia and dried overnight at room temperature. Sections were incubated with monoclonal antibodies specific for recombinant human granzyme B[27] for 60 minutes. In the control sections, the primary antibody was omitted or irrelevant antibodies were applied (isotype-matched anti-human immunodeficiency virus antibody, a gift from TNO, Rijswijk, The Netherlands). Subsequently, horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Dako, Glostrup, Denmark) was added, followed by incubation with biotinylated tyramide[28] and administration of streptavidine-HRP (Zymed, San Fransisco, CA), each incubation step lasted 30 min. HRP activity was detected using hydrogen peroxide as substrate and aminoethylcarbazole (Sigma, St. Louis, MO). Slides were counterstained with Mayer's Hämalaunlösung (Merck) and, after washing with distilled water, mounted in Kaiser's glycerol gelatine (Merck). The sections were washed between all steps with PBS and all incubations were carried out at room temperature.

Calculations and statistical analysis

All experiments were performed in triplicate. The results are given as the mean and standard error of the mean. Differences between levels of degradation were calculated with two-sided Student's t-test for independent samples.

Results

Incubation with granzyme B results in release of newly synthesised proteoglycans of the chondrocyte extracellular matrix and leaves newly synthesised collagen unaffected.

Incubation with 0.4 μg/ml granzyme B induced the release of the ^{35}S sulfate-labeled matrix components after 24 h. Release of approximately 30% above the background level of ^{35}S from the matrix was observed. Granzyme B in a concentration of 0.04 μg/ml did not induce degradation of ^{35}S-sulfate labeled matrix (Figure 1; black bars).

In contrast, granzyme B did not induce the release of ^{3}H proline-labeled collagen after 24 h of incubation at any of the concentrations tested (Figure 1; hatched bars).

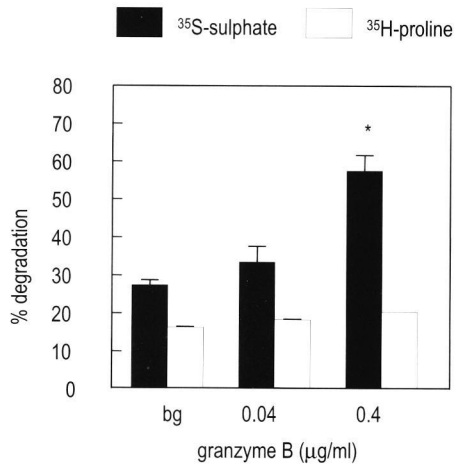

Figure 1. *Degradation of ^{35}S sulfate-labeled and of ^{3}H-proline-labelled cartilage matrix cultured in alginate beads for 4 weeks after incubation with 0.04 and 0.4 μg/ml human granzyme B during 24 hours (black and white bars respectively). The background levels (bg) indicate the spontaneous release of ^{35}S and ^{3}H in the medium without addition of granzyme B. Results are mean and standard error of the mean of radioactivity released in the culture medium as a percentage of the total radioactivity (n=3). * = $p<0.05$ (Student's t-test for independent samples).*

Granzyme B degrades resident proteoglycans and not collagen of cartilage explants. Bovine articular cartilage explants were incubated with 1 ml of 0.04 and 0.4 µg/ml Gran B for 15 and 90 h. Fifteen hours of incubation with 0.4 µg/ml granzyme B resulted in a significant increase of GAG release up to 3.3 µg/mg cartilage (figure 2; black bars). After 90 hours of incubation, both 0.04 µg/ml and 0.4 µg/ml granzyme B increased GAG release to 6.1 and 11.7 µg/mg cartilage, respectively (Figure 2; shaded bars).

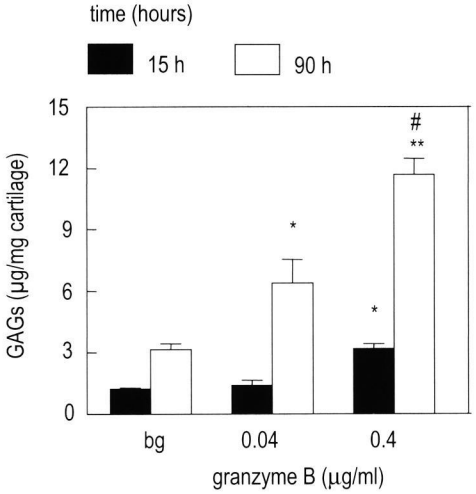

Figure 2. *Release of GAGs from bovine articular cartilage explants after incubation with 0.04 and 0.4 µg/ml human granzyme B during 15 and 90 h (black and white bars respectively). Results are given as the mean and standard error of the mean GAG (µg) released in the culture medium per mg cartilage (n=3). * = $p<0.05$, ** = $p<0.01$ as compared to background levels at the same time point; # = $p<0.05$ as compared to levels with 0.04 µg/ml (Student's t-test for independent samples).*

No collagen degradation could be detected after 90 hours of incubation with either concentrations of granzyme B; hydroxyproline release did not rise above background levels (Figure 3; black bars) and no increase in the amount of denatured collagen was found in the explants (figure 3; white bars).

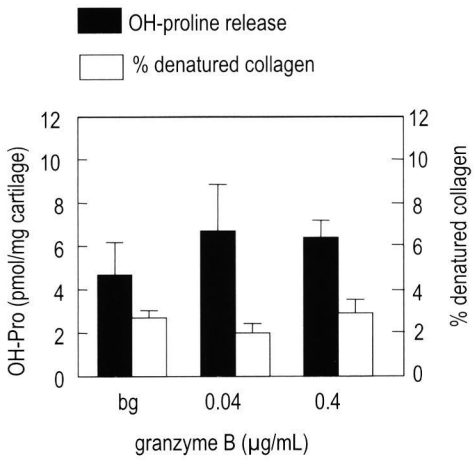

Figure 3. *The black bars show the release of hydroxyproline from bovine articular cartilage explants after incubation with 0.04 and 0.4 µg/ml human granzyme B during 90 hours. Results are given as the mean and standard error of the mean hydroxy (OH-) proline (pmol) released in the culture medium per mg cartilage. The white bars show the fraction of denatured collagen after incubation with 0.04 and 0.4 µg/ml human granzyme B during 90 hours as the percentage of total collagen (mean and standard error of the mean).*

Localization of granzyme B positive cells at the pannus-hard tissue junction.
Immunohistologic analysis of plastic-embedded sections of metacarpophalangeal joints from patients with rheumatoid arthritis yielded granzyme B-positive cells throughout the synovium (figure 4A) and also, although not in massive numbers, at the invasive front of the pannus tissue (figure 4B). Granzyme B was found in granules both inside and outside the cells (figure 4A). Staining was negative when the primary antibody was omitted or irrelevant antibodies were applied as controls.

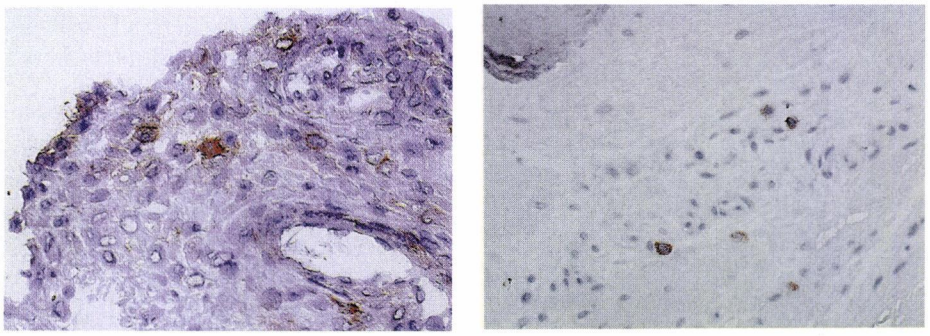

Figure 4. Panel A. Granzyme B is present throughout the rheumatoid synovium in granules, both inside and outside the cells. Panel B. Localisation of granzyme B positive cells at the pannus-hard tissue junction in metacarpophalangeal joints of patients with rheumatoid arthritis. (Single-stain peroxidase technique; Mayer's Hämalaunlösung counterstained; original magnification x 250). Note the bone tissue in the top left hand corner.

Discussion

RA is a progressive, destructive disease affecting metacarpophalangeal and metatarsophalangeal joints most frequently. Within the joint, the pannus tissue overgrows and invades the underlying cartilage and bone. This tissue destruction involves both damage to the collagen fibrillar network as well as loss of proteoglycans.[29,30] Cartilage degradation primarily results from the action of extracellular proteolytic enzymes produced by many cell types in response to microenvironmental factors. Several classes of proteinases are responsible for tissue destruction in RA. Much emphasis has been placed on serine proteinases and matrix metalloproteinases.[30-35] Recently, granzymes have also been suggested to play a role in the degradation of extracellular matrix.[11,17,36] Granzymes are serine proteases that are found in granules in cytotoxic T cells and natural killer cells.[19,20] Levels of granzyme A and B are specifically increased in synovial fluids of RA patients as compared to synovial fluids of patients with osteoarthritis or reactive arthritis.[17] In RA synovial tissue, a significantly higher number of granzyme B-positive cells was found compared with synovial tissue of patients with reactive arthritis.[36] When granzyme-containing granules are released from the cells, they

may exert extracellular activity, such as proteolysis of extracellular matrix components. Granzyme A was shown to be capable of degrading collagen type IV from the basement membrane.[13] Froelich *et al.*[11] provided evidence for aggrecan-degrading activity of granzyme B. The objectives of the present study were to further explore the cartilage degrading properties of granzyme B and to investigate the presence of granzyme B at the invasive front of the pannus tissue.

The capacity of granzyme B to degrade articular cartilage matrix was assessed *in vitro*. The availability of purified human granzyme B provided the opportunity to directly investigate the capacity of this enzyme to degrade an organized newly synthesized cartilage matrix, produced during 4 weeks of chondrocyte culture, and to degrade explants of intact bovine articular cartilage. Incubation with granzyme B resulted in the loss of proteoglycans from the newly synthesised cartilage matrix. These results are in line with the findings of Froelich *et al.*,[11] indicating that granzyme B is indeed capable of directly degrading newly synthesised proteoglycans. Granzyme B also mediated the release of glycosaminoglycans from whole articular cartilage explants, suggesting the digestion of proteoglycans to protein fragments which are small enough to diffuse out of the tissue.

In contrast to its proteoglycan-degrading activity, we found no collagenolytic activity of granzyme B in our models. No significant release of ^{3}H proline could be detected from the newly synthesised cartilage matrix, neither was granzyme B able to release hydroxyproline from the articular cartilage explants or to cause the denaturation of resident collagen. Although granzyme A is able to degrade preferentially the α2 chain of basement membrane type IV collagen,[13] our results indicate that granzyme B is not capable of degrading the collagen component of articular cartilage in our *in vitro* models. This however, does not exclude the possibility that, *in vivo, g*ranzyme B acts in concert with other proteinases to cause collagen degradation e.g. by making collagen more susceptible degradation by other proteases, or by activating other pro-enzymes.

The effect of granzyme B on glycosaminoglycan release from the cartilage explants was consistent with the effect on ^{35}S sulfate release from the newly synthesized cartilage matrix. Likewise, the lack effect of granzyme B on hydroxyproline release from the cartilage explants, indicating collagen degradation, was consistent with the lack of effect on ^{3}H proline release from the cartilage matrix. This strongly suggests that ^{35}S sulfate is mainly incorporated in the proteoglycans and that ^{3}H proline is mainly incorporated, probably as ^{3}H hydroxyproline, in the collagen fraction of the cartilage matrix. This allows the use of this sensitive cartilage degradation model to distinguish between the effects of proteolytic enzymes on proteoglycan and collagen degradation.

To investigate the possibility that granzyme B is involved in cartilage degradation in RA, we investigated the presence of granzyme B at the invasive front of the pannus tissue. Expression of granzyme B has been found in synovial tissue.[12] These granzyme B-positive cells were seen in the synovial lining and in the sublining area, but the presence of these cells at the particular site where erosions develop was never investigated. In the present study we

found granzyme B-positive cells throughout the synovial tissue and, although not in massive numbers, at the invasive front of the pannus tissue. We found granzyme B-containing granules both inside and outside the cells. The release of granzyme B-containing granules at the invasive front of the pannus tissue may lead to the active binding of positively charged granzyme B to the anionic extracellular matrix and, in the apparent absence of an endogenous inhibitor, to local concentrations effective to degrade proteoglycans. The identity of the granzyme B positive cells could not be studied directly by double staining for technical reasons as the use of plastic to embed our sections resulted in non-specific adsorption of certain antibodies. However, since only cytotoxic lymphocytes and natural killer cells are able to express this enzyme,[19,20] the finding of positive staining for granzyme B indicates the presence of these cell types in the invading synovial tissue. The previously demonstrated increased numbers of these granzyme B-positive cells and the their location suggests that cytotoxic lymphocytes and natural killer cells may be involved in matrix degradation in several ways: by cell-cell interactions, by cytokine-mediated stimulation of matrix degrading synoviocytes, and by the local production of matrix degrading enzymes, including granzyme B.

The results of the present study confirm that human granzyme B is capable of degrading the proteoglycan component of cartilage. This finding, together with the presence of granzyme B at the invasive front of the pannus tissue and the previously observed increased expression of granzyme B in rheumatoid synovial joints suggest that granzyme B is involved in the destruction of articular cartilage in RA.

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4

CARTILAGE DEGRADATION AND INVASION BY RHEUMATOID SYNOVIAL FIBROBLASTS IS INHIBITED BY GENE TRANSFER OF A CELL SURFACE-TARGETED PLASMIN INHIBITOR

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Arthritis & Rheumatism 2000;43:1710-1718

Summary

Joint destruction in rheumatoid arthritis (RA) is a result of degradation and invasion of the articular cartilage by the pannus tissue. In the present study, the role of the plasminogen activation system in cartilage degradation and invasion by synovial fibroblasts and a novel gene therapeutic approach were investigated using a cell surface-targeted plasmin inhibitor (ATF.BPTI).

Adenoviral vectors were used for gene transfer. The effects of ATF.BPTI gene transfer on rheumatoid synovial fibroblasts-dependent cartilage degradation were studied *in vitro* and cartilage invasion was studied *in vivo* in the SCID mouse co-implantation model.
The results indicate that cartilage matrix degradation by rheumatoid synovial fibroblasts is plasmin mediated and depends on urokinase-type plasminogen activator (uPA) for activation. Targeting plasmin inhibition to the cell surface of the fibroblasts by gene transfer of a cell surface-binding plasmin inhibitor resulted in a significant reduction of cartilage matrix degradation *in vitro* and cartilage invasion *in vivo*. Compared with uninfected rheumatoid synovial fibroblasts, the mean ± SEM cartilage degradation *in vitro* was reduced 87.9 ± 0.9% after LacZ gene transfer versus a reduction of 24.0 ± 1.6% after ATF.BPTI gene transfer ($p<0.0001$). The mean ± SEM i*n vivo* cartilage invasion score was 3.1±0.4 in the control-transduced fibroblasts and 1.8 ± 0.4 in the ATF.BTPI-transduced fibroblasts ($p<0.05$).

These results indicate a role of the PA-system in synovial fibroblast-dependent cartilage degradation and invasion in RA, and demonstrate an effective way to inhibit this by gene transfer of a cell surface-targeted plasmin inhibitor.

Introduction

Invasion of articular cartilage by the inflamed and hyperplastic synovial (pannus) tissue represents a hallmark of joint destruction in rheumatoid arthritis (RA).[1] Activated synovial fibroblasts have been shown to be intimately involved in this destructive process by their attachment to the cartilage surface and the release of matrix degrading enzymes.[2] These locally secreted proteolytic enzymes degrade the articular cartilage at the pannus-cartilage interface, providing the space for the pannus tissue to invade. Although all classes of proteolytic enzymes seem to be involved, cartilage destruction has mainly been attributed to matrix metalloproteinases (MMPs) and serine proteases.[3]

Among the serine proteases, plasmin and plasminogen activators may be of interest, because of their fibrinolytic function, their capacity to degrade a wide variety of extracellular matrix proteins and their capability to activate latent forms of collagenases and stromelysin.[4] In rheumatoid synovium, components of the plasminogen activation (PA)-system and its inhibitors are expressed in significantly higher amounts compared with osteoarthritic and non-arthritic synovium.[5] These components are mainly located at the hyperplastic synovial lining, the invasive front of the pannus tissue. In comparison with non-arthritic synovial fibroblasts, increased production of urokinase-type plasminogen activator (uPA) the uPA-receptor (uPAR) ,[6] and proteins involved in plasminogen binding is observed in rheumatoid synovial fibroblasts.[7] Findings of *in vivo* investigations of the effects of plasmin inhibition in experimental arthritis and RA support a role of the plasminogen activation system in joint destruction.[8]

The hyperplasia and invasive growth of the pannus tissue in RA resemble the invasive behavior of tumor cells. In several malignancies, the PA-system has been implicated as an important mediator of cellular invasion and metastasis.[9] The possibility that the PA-system is involved in cartilage degradation and invasion by the pannus tissue in RA is explored in the present study.

A well-described model for studying the invasion of articular cartilage by rheumatoid synovial fibroblasts is the severe combined immunodeficient (SCID) mouse coimplantation model.[2] In this model, rheumatoid synovial fibroblasts are co-implanted with human articular cartilage under the renal capsule of SCID mice for a period of 60 days. Since inflammation is absent in this model, the destructive process in itself can be investigated. This model is very useful for studying the effect of gene transfer on invasion, as gene expression during a period of >2 months has been described.[10]

The first question we addressed in this paper is whether the plasminogen activation system mediates cartilage degradation by rheumatoid synovial fibroblasts *in vitro*. Second, we investigated a novel gene therapeutic strategy using a cell surface-targeted plasmin inhibitor to inhibit plasmin-mediated cartilage degradation and invasion by rheumatoid synovial fibroblasts both *in vitro* and *in vivo*.

Materials and Methods

Adenoviral vectors (Figure 1).

Replication-defective adenoviral vectors (Ad; E1-deleted, cytomegalovirus promotor) were used. To study the effects of cell surface-targeted plasmin inhibition, we used an Ad encoding ATF.BPTI, a hybrid protein consisting of a plasmin inhibitor, bovine pancreatic trypsin inhibitor (BPTI), linked to the receptor-binding aminoterminal fragment (ATF) of uPA. Vectors encoding BPTI or ATF only were used to compare the effects of ATF.BPTI with those of a nonbinding plasmin inhibitor (BPTI) and of a noninhibiting receptor-binding protein (ATF). The construction of AdATF.BPTI, AdBPTI and AdATF is described in detail elsewhere.[11] Identical vectors either lacking and insert or encoding beta-galactosidase (β-gal) were used as control vectors. AdLacZ, a beta-galactosidase-encoding adenoviral vector, was a kind gift of Dr R. Hoeben (Leiden University Medical Center, Leiden, The Netherlands).

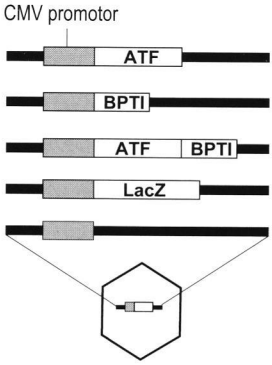

Figure 1. E1-deleted adenoviral vectors (Ad) used in this study. *Vectors encoding the cell surface-binding aminoterminal fragment of uPA (AdATF), bovine pancreatic trypsin inhibitor (AdBPTI), the cell surface-binding plasmin inhibitor (AdATF.BPTI) consisting of ATF linked to BPTI, or betagalactosidase (AdLacZ) and a control vector without an insert were used.*

Isolation and culture of rheumatoid synovial fibroblasts.

Synovial tissues were obtained at joint surgery with informed consent from patients with active RA according to the revised criteria of the American College of Rheumatology (formerly, the American Rheumatism Association).[12] RA synovial fibroblasts were isolated by collagenase digestion and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco Life Technologies, Paisley, UK)/Ham's F12 medium (BioWhittaker, Verviers, Belgium) supplemented with 10% (volume/volume) newborn calf serum (NCS), 10% (v/v) normal human serum (NHS), 100 IU/ml penicillin and 100 μg/ml streptomycin (Boehringer, Mannheim, Germany) at 37°C in a 5% CO_2 humidified atmosphere. Synovial fibroblasts used in the SCID mouse coimplantation model were cultured in DMEM/F12 with 10% fetal calf serum (FCS). Synovial fibroblasts of several RA patients up to the third passage were used for the *in vitro* experiments. In the SCID mouse model, the synovial fibroblasts of a single donor were used (fourth passage).

Transduction efficiency.

Rheumatoid synovial fibroblasts (5×10^4/well) were incubated with 3×10^6, 1.8×10^7, 3×10^7, 1.8×10^8, or 3×10^8 plaque-forming units (PFU) AdLacZ in 300 ml DMEM/F12 with 5% NCS for 3 hours and cultured in fresh DMEM/F12 with 10%HS and 10% NCS for 48 hours. A beta-gal staining was performed to assess the percentage of transduced cells.[13]

Plasmin inhibitory activity after ATF.BPTI and BPTI gene transfer.

To establish whether ATF.BPTI and BPTI show plasmin inhibitory activity, as well as whether ATF.BPTI gene transfer produces cell surface-bound plasmin inhibition, rheumatoid synovial fibroblasts (5×10^4 / well) were infected with AdATF.BPTI, AdBPTI, or AdLacZ at a concentration of 3×10^7 PFU in 300 µl serum-free DMEM/F12 for 3 hours and subsequently cultured in 300 µl fresh serum-free DMEM/F12 for 24 hours. Conditioned medium was collected for BPTI activity measurement. To study plasmin inhibitory activity at the cell surface, we subsequently treated the cells with 50 mM glycin. HCl (pH 3) in 100 mM NaCl for 3 minutes to detach any molecules bound to the uPAR. The glycin fraction, i.e. the uPAR-bound fraction, was neutralized to a pH of ~7.5. Conditioned medium and glycin dilutions were incubated for 15 minutes at room temperature with plasmin (88 pM). A chromogenic substrate (0.1 mM; S2251; Chromogenix, Möndal, Sweden) was added and after 24 hours of incubation at 37°C, the absorbance at 405 nm was measured to determine the conversion of S2251. BPTI (Trasylol®, Bayer, Leverkusen, Germany) in concentrations of 0.004-0.5 kallikrein inhibiting units (KIU)/ml were used as reference values.

Long-term expression of ATF.BPTI by rheumatoid synovial fibroblasts in culture.

Rheumatoid synovial fibroblasts (1×10^5) were seeded in a 25-cm^2 culture flask and incubated with 1×10^8 PFU AdATF.BPTI in 5 ml DMEM/F12 with 5% NCS for 24 hours and subsequently cultured during 6 weeks in DMEM/F12 with 10% NHS and 10% NCS. Once each week, 24-hours medium was collected for enzyme-linked immunosorbent assay (ELISA) measurements of ATF.BPTI and the cells were subsequently passaged to 3 25-cm flasks.

Production of radiolabeled chondrocyte-synthesized extracellular matrix.

A cartilage-like matrix was produced by culturing chondrocytes in alginate beads as described previously.[14,15] During 28 days, 0.25 µCi/ml ^{3}H-Proline (Amersham Pharmacia Biotech, Amersham, UK) was added twice each week to fresh medium. Thereafter, the alginate beads were dissolved in 55 mM sodium citrate to release the matrix. The solution was centrifuged (750 g, 6 minutes), the matrix pellet was suspended in DMEM, and the matrix was coated for 3 days at 4°C in 24-well plates, fixed with 500 µl methanol, and air-dried. Plates were stored at –20°C until use.

Degradation of ^{3}H-Proline labeled cartilage matrix.

The role of the plasminogen activation plasmin system in cartilage degradation by rheumatoid synovial fibroblasts was investigated in several steps. The rheumatoid synovial fibroblasts (5×10^4/well) were seeded on the matrix in DMEM/F12 with 10% NHS and 10% NCS in the presence of 0.15 µM plasminogen and the following inhibitors of plasmin and the PA-system: a neutralizing monoclonal antibody against human uPA (anti-uPA; 15 µg/ml),[16] a neutralizing polyclonal antibody against human tissue-type plasminogen activator (anti-tPA; 20 µg/ml),[16] a monoclonal antibody blocking uPA binding to the uPA-receptor (anti-uPAR; 100 µg/ml, a kind gift from Dr. U. Weidle, Boehringer Mannheim, Penzberg, Germany), and serine protease inhibitor BPTI (Trasylol®, Bayer, Leverkusen, Germany; 100 KIU/ml).

To study the effects of gene transfer aimed at inhibition of the PA-system, rheumatoid synovial fibroblasts (5×10^4 / well) were seeded on top of the matrix in DMEM/F12 with 5% NBCS and incubated with 3×10^7 PFU of AdATF.BPTI, AdBPTI, AdATF, or AdLacZ in 300 µl final volume for 3 hours, and subsequently cultured in fresh DMEM/F12 with 10% NHS and 10% NCS. At 24 hours 0.15 µM plasminogen was added. After 72 hours of incubation at 37°C in a humidified atmosphere of 5% CO_2, the supernatants were collected for ELISA measurements and ^{3}H-Proline release measurements by a liquid scintillation analyzer (Tri-Carb 1900, Packard, Ca). Residual matrices were dissolved overnight in 0.1 M NaOH for ^{3}H-Proline measurements. The percentage ^{3}H-Proline release (of the total ^{3}H-Proline present) in the medium was used as a measure for cartilage matrix degradation. Cartilage matrix degradation by the cells treated with an inhibitor or with gene transfer is expressed as the percentage of cartilage matrix degradation by the control rheumatoid synovial fibroblasts. The results are presented as mean and SEM. Two-tailed Student's 2-tailed t-tests were performed to determine significant effect differences due to different conditions.

SCID mouse co-implantation model.

Rheumatoid synovial fibroblasts (5×10^5) were seeded in a 75-cm^2 culture flasks and were incubated for 24 hours with 1.5×10^8 pfu of either the ATF.BPTI encoding construct or the control construct in 15 ml DMEM/F12 with 5% FCS. After 24 hours, the cells were washed and incubated for 24 hours with DMEM/F12 with 10% FCS. At 48 hours after transduction, fibroblasts were trypsinized, washed, spun down, and resuspended in 250 µl DMEM/F12 with 10% FCS and inserted into 2-mm^3 sterile Gelfoam sponges (Pharmacia & Upjohn, Kalamazoo, MI, USA) as described previously.[2] Fresh articular cartilage of the knee was obtained from the pathology department and cut in 2-3 mm^3 pieces. Under sterile conditions, the sponges containing the transduced fibroblasts were co-implanted with the cartilage pieces under the renal capsule in the SCID mice. A total of 16 mice were used for the implantations: 8 were implanted with ATF.BPTI-transduced fibroblasts and 8 received fibroblasts infected by the control construct. After 60 days, the implants were removed and paraffin sections were made. Histologic evaluation of the sections was performed as described previously.[17]

Differences between groups were tested for statistical difference using the Mann-Whitney U-test.

ELISAs.

Production of uPA and tPA in conditioned medium of cultured synovial fibroblasts was measured by ELISA.[18,19] To compare ATF and ATF.BPTI expression, another uPA specific ELISA recognizing the ATF fragment of uPA was performed in the conditioned medium of ATF- or ATF.BPTI-transduced rheumatoid synovial fibroblasts.[11] Endogenous uPA production (in the order of ng/ml) by the synovial fibroblasts did not influence the ATF and ATF.BPTI measurements since ATF and ATF.BPTI production by the synovial fibroblasts after infection was a 1,000-fold higher. The results are presented as mean and SEM. To compare ATF.BPTI and BPTI production, a BPTI ELISA was performed.[11] BPTI levels are expressed as KIU/ml.

uPA receptor assessments.

The presence of the uPA receptor on synovial cells was determined using a cross-linking assay.[20]

Cell viability assessment.

Rheumatoid synovial fibroblasts were seeded in a 24 well plate (3.5×10^4 cells/well) and incubated with 3×10^6, 1.8×10^7, 3×10^7, 1.8×10^8, 3×10^8 PFU AdATF.BPTI in 300 µl DMEM/F12 with 5% NBCS. After 3 hours, the cells were washed and were incubated in serum-free DMEM/F12 for 40 hours. After 40 hours, the cells were washed and incubated for 3 hours in 37°C with an MTT solution (1 mg MTT/ml PBS; Sigma Chemical Company, St Louis, MO). After 3 hours the cells were lysed. Of the lysates 100 µl was transferred to an ELISA-plate and extinction was measured at 540 nm.

Results

Plasmin-mediated, uPA-dependent degradation of cartilage-like matrix by rheumatoid synovial fibroblasts.

PA production by rheumatoid synovial fibroblasts was studied by analysis of the conditioned medium. Rheumatoid synovial fibroblasts predominantly produced uPA (up to a level of 8 ng/ml after 96 hours of culture) but hardly any tPA (< 1 ng/ml) as measured by ELISA. The uPAR was present on the cells as determined by cross-linking experiments with radiolabeled uPA. To study the role of plasmin, uPA, tPA, and uPAR in synovial fibroblast-dependent cartilage degradation, we cultured rheumatoid synovial fibroblasts on a radiolabeled cartilage-like matrix in the presence of plasminogen and inhibitors. In the presence of plasminogen, but in the absence of synovial fibroblasts, only a little degradation took place ($7.3 \pm 2.2\%$). In the

presence of rheumatoid synovial fibroblasts, 30-60% of the matrix was degraded as measured by ^{3}H release into the medium after 72 hours (Figure 2). Synovial fibroblast-dependent matrix degradation was blocked in a dose-dependent manner by BPTI ($p < 0.0001$; $n = 7$; Figures 2 and 3). Anti-uPA caused a 44.2 ± 3.2% inhibition of matrix degradation ($p < 0.05$; $n = 3$), whereas anti-tPA had no effect (Figure 2).

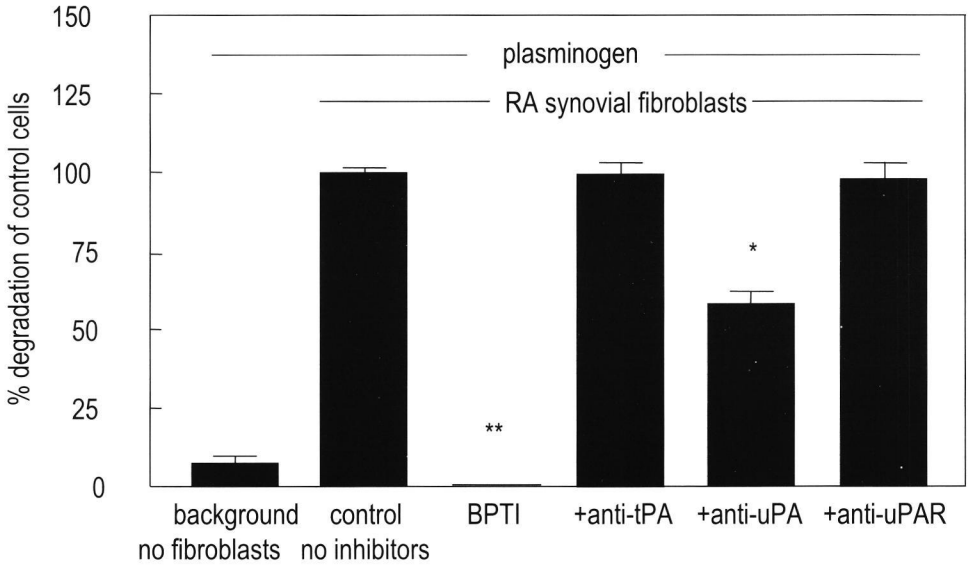

Figure 2. Effects of inhibiting plasmin and plasminogen activators (PA) on in vitro degradation of a 3H-labeled cartilage matrix by rheumatoid (RA) synovial fibroblasts cultured for 72 hours in the presence of 0.15 μM plasminogen. *Cartilage matrix degradation by RA synovial fibroblasts is shown as a percentage of cartilage matrix degradation by the control cells (RA synovial fibroblasts in the absence of inhibitors). The percentage of ^{3}H release by RA synovial fibroblasts in the absence of inhibitors is defined as 100% degradation. Shown are the effects of adding plasmin inhibitor bovine pancreatic trypsin inhibitor (BPTI; 100 kallikrein-inhibiting units/ml), a neutralizing polyclonal antibody against tissue type PA (anti-tPA; 20 μg/ml), a neutralizing monoclonal antibody against human urokinase-type PA (anti-uPA; 15 μg/ml), and a monoclonal antibody blocking uPA binding to the uPA receptro (anti-uPAR; 100 μg/ml) to the synovial fibroblast culture. Values are the mean and SEM. * = $p<0.05$; ** = $p<0.0001$, versus control.*

Infection efficiency and toxicity after adenoviral transduction of synovial fibroblasts.
The efficiency of adenoviral infection of synovial fibroblasts in culture conditions (5×10^4/well) was determined with a beta-galactosidase encoding adenovirus (AdLacZ). Rheumatoid synovial fibroblasts were incubated with 3×10^6, 1.8×10^7, 3×10^7, 1.8×10^8, or 3×10^8 pfu AdLacZ in 300 µl for 3 hours, yielding transduced-cell percentages of 5%, 30%, 80%, 100%, and 100%, respectively. Infection with concentrations of 1.8×10^8 PFU adenovirus resulted in changes in cell morphology and a concentration of 3×10^8 PFU adenovirus even reslulted in cell death, as assessed by light microscopy. A cell viability test did not show a decrease in mitochondrial enzyme activity up to virus concentrations of 3×10^7 PFU. Expression of the ATF.BPTI protein showed an increase up to 1.8×10^8 PFU AdATF.BPTI, and a decrease at higher concentrations. To prevent an inhibitory effect due to toxicity of adenoviral gene transfer, we chose a concentration of 3×10^7 PFU / 300 µl for further experiments.

Gene expression after ATF.BPTI, BPTI and ATF gene transfer.
ATF.BPTI, ATF, and BPTI levels were measured in the conditioned medium of ATF.BPTI-, ATF-, or BPTI-transduced synovial fibroblasts in the degradation experiment by ELISA. At 72 hours after gene transfer, 19.1 ± 2.2 µg/ml ATF.BPTI and 81.3 ± 0.3 µg/ml ATF were measured by uPA-ELISA. BPTI-ELISA showed 0.21 ± 0.01 KIU/ml ATF.BPTI and 0.22 ± 0.01 KIU/ml BPTI.

Plasmin inhibitory activity after ATF.BPTI and BPTI gene transfer.
BPTI activity was determined by a plasmin inhibition assay in both the culture supernatants and the cell surface-bound fraction of AdATF.BPTI- and AdBPTI-transduced synovial fibroblasts cultured for 24 hours in serum-free conditions. In the culture supernatants of AdATF.BPTI- and AdBPTI-transduced cells, plasmin inhibitition activity levels of 2.0 ± 0.1 and 1.9±0.5 KIU/ml, respectively, were measured. A plasmin inhibition activity level of 0.5±0.2 KIU/ml was observed at the cell surface of ATF.BPTI-transduced synovial fibroblasts, was observed, while no plasmin inhibition activity was observed at the cell surfaces of AdBPTI-transduced synoviocytes.

Duration of gene expression in culture.
Until 6 weeks after gene transfer, the production and secretion of ATF.BPTI remained at constant levels as measured by ELISA.

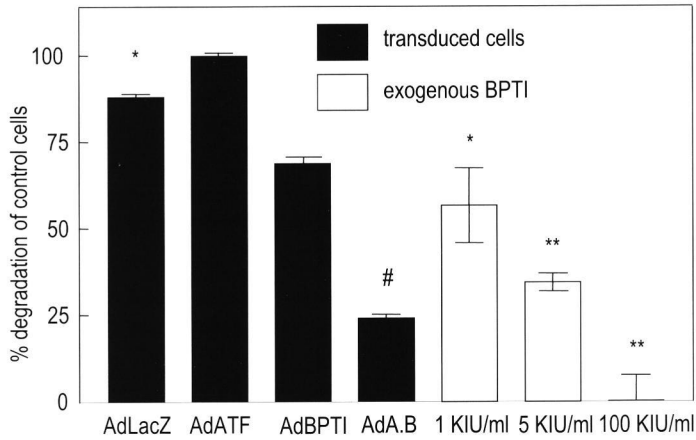

Figure 3. Effects of transduction with AdBPTI, AdATF.BPTI, AdATF, or AdLacZ on* in vitro *degradation of a ^{3}H-labeled cartilage matrix by RA synovial fibroblasts cultured for 72 hours in the presence of 0.15 μM plasminogen. *Cartilage matrix degradation by RA synovial fibroblasts after adenoviral infection or after addition of exogenous BPTI is shown as a percentage of cartilage matrix degradation by the uninfected RA synovial fibroblasts. AdLacZ was used as a control vector. Values are the mean ± SEM. * = $p<0.05$ and ** = $p<0.0001$ compared with uninfected syovial fibroblasts; # = $P<0.0001$ compared with AdLacZ-transduced synovial fibroblasts. KIU = kallikrein-inhibiting units. See Figure 1 for other definitions.*

Effects of ATF.BPTI and BPTI gene transfer degradation of a cartilage-like matrix by rheumatoid synovial fibroblasts.

Rheumatoid synovial fibroblasts were transduced with AdATF.BPTI, AdBPTI, AdATF, or AdLacZ to investigate the effect of adenoviral gene transfer on plasmin-mediated cartilage matrix degradation. BPTI gene transfer resulted in a 31.3 ± 3.0% reduction of cartilage matrix degradation compared with uninfected synovial fibroblasts. Compared with AdLacZ-transduced synovial fibroblasts, this did not reach statisitical significance ($n = 8$; $p = 0.06$) (Figure 3). This effect was comparable with that of adding 1 KIU/ exogenous BPTI of (Figure 3), while the level of BPTI measured in the medium by ELISA was only 0.2 KIU/ml. Targeting plasmin inhibition at the cell surface by transduction with AdATF.BPTI resulted in a 76.0 ± 1.6% reduction of cartilage matrix degradation compared with uninfected synovial fibroblasts ($p < 0.0001$ versus AdLacZ; $n = 13$) at a measured ATF.BPTI level in the medium of 0.2 KIU/ml. This effect was comparable with that observed after addition of 5 KIU/ml exogenous BPTI (Figure 3). Gene transfer of ATF, the non-inhibiting receptor-binding

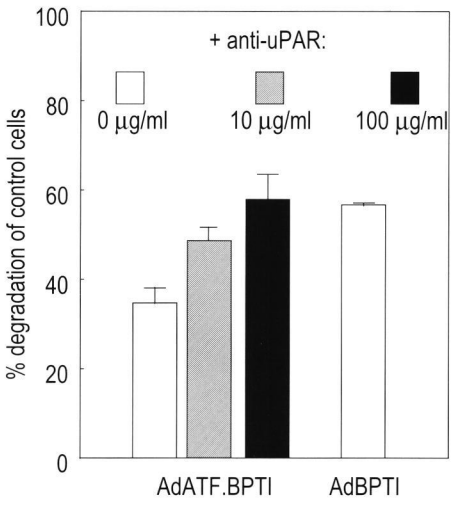

Figure 4. Effects of blocking a plasminogen activator receptor (PAR) on in vitro degradation of a ^{3}H-labeled cartilage matrix by rheumatoid synovial fibroblasts cultured for 72 hours in the presence of 0.15 µM plasminogen. *Binding of ATF.BPTI to the human urokinase-type PAR (uPAR) was blocked by adding a monoclonal antibody (anti-uPAR), resulting in dose-dependent suppression of cartilage matrix degradation inhibition after ATF.BPTI gene transfer. Shown is the percentage of cartilage matrix degradation by RA synovial fibroblasts after adenoviral infection and addition of anti-uPAR compared with the control. Values are the mean ± SEM. See Figure 1 for other definitions.*

fragment, did not inhibit cartilage degradation at all (n = 7). Infecting the cells with AdLacZ resulted in a slight reduction of cartilage matrix degradation compared with uninfected synovial fibroblasts (12.1 ± 0.9%; $p < 0.05$; n = 10) (Figure 3).

The contribution of targeting plasmin inhibition to the cell surface was investigated by inhibiting the receptor binding with a monoclonal antibody against the uPAR (anti-uPAR). Rheumatoid synovial fibroblasts were cultured in the presence of anti-uPAR before, during and after infection with AdATF.BPTI. The addition of anti-uPAR induced a dose dependent suppression of inhibition after ATF.BPTI gene transfer toward degradation levels observed after BPTI gene transfer (Figure 4).

Effects of ATF.BPTI gene transfer on cartilage invasion by rheumatoid synovial fibroblasts in vivo.

Rheumatoid synovial fibroblasts were infected with AdATF.BPT, or with an identical control construct without the ATF.BPTI-encoding gene and co-implanted under the renal capsule of a SCID mouse for 60 days. Histologic evaluation showed a significant reduction of the invasiveness into the cartilage of the ATF.BPTI-transduced synovial fibroblasts compared with the control synovial fibroblasts. Deep invasion (an invasion score of ≥ 2.5) was observed in 7 out of 8 cartilage sections from the mice implanted with the fibroblasts infected with the control construct (Figure 5A and B).

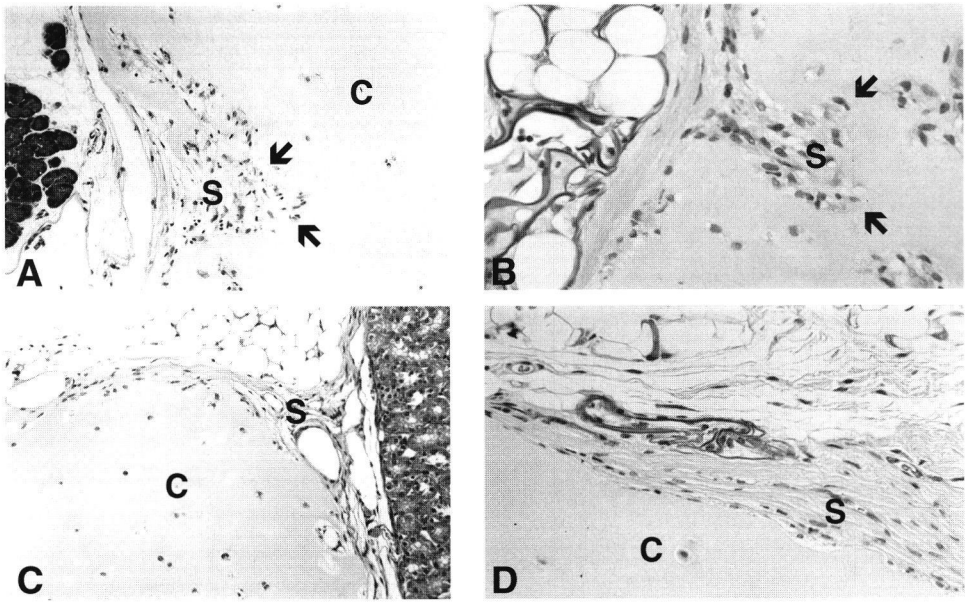

Figure 5. Coimplantation of AdATF.BPTI-transduced and control synovial fibroblasts (S) with human articular cartilage (C) under the renal capsule of SCID mice. *After 60 days, the coimplants were removed, stained by hematoxylin and eosin, and evaluated histologically. A and B: sections with control synovial fibroblasts. C and D: sections with ATF.BPTI transduced synovial fibroblasts. In A and B, the control synovial fibroblasts have deepl invaded the cartilage. Arrows show the invasive front of the synovial fibroblasts. In C and D, AdATF.BPTI transduced synovial fibroblasts accumulate at the edge of the cartilage, bu hardly any invasion is observed. (Original magnification × 200 in A and C; and × 400 in B and D.) See Figure 1 for more definitions.*

In contrast, we observed deep invasion in only 1 out of 8 cartilage sections from the mice implanted with the AdATF.BPTI-transduced synovial
fibroblasts; the other sections showed hardly any, or limited, invasion (score < 2.5) (Figure 5C and D). The mean ± SEM invasion score in cartilage sections with AdATF.BPTI-transduced fibroblasts were 1.8 ± 0.4, compared with 3.1 ± 0.4 in sections with the control fibroblasts ($p < 0.05$; $n = 8$ per group (Figure 6A). No effect of ATF.BPTI gene transfer on pericellular chondrocyte-dependent cartilage degradation was observed (Figure 6B).

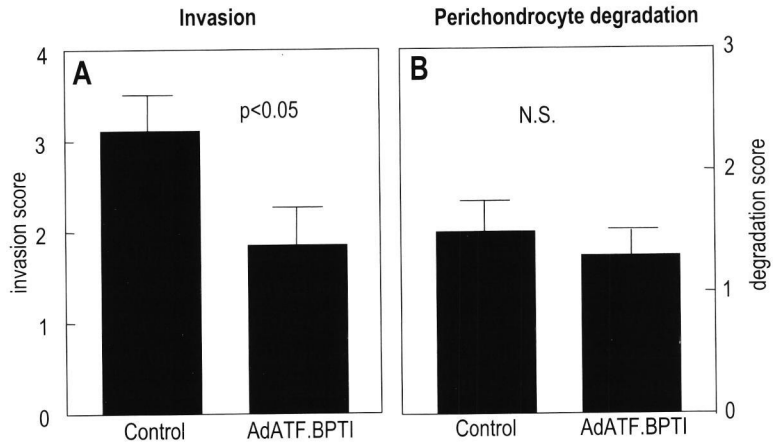

Figure 6. ATF.BPTI gene transfer inhibition of cartilage invasiob and chondrocyte-dependent cartilage degradation (Perichondrocyte degradation). *Values are the mean and SEM. A: Cartilage invasion by AdATF.BPTI-transduced RA synovial fibroblasts was scored in sections of the coimplants from the SCID mice and compared with the invasion scores of synovial fibroblasts transduced by a control vector. ATF.BPTI gene transfer significantly inhibited cartilage invasion ($p<0.05$; $n=8$ per group). B. Perichondrocyte degradation was not inhibited by ATF.BPTI gene transfer (N.S. = not significant). See Figure 1 for other definitions.*

Discussion

Joint destruction in RA is a result of the invasive growth of the pannus tissue in to the articular cartilage. The activated synovial fibroblast is considered to be directly involved in this process.[21] Knowledge about the mechanisms that cause cartilage destruction in RA may lead to novel targets for therapy. The observation of increased plasminogen activation in the pannus tissue in the joints in RA,[5,22] and the observed effects of plasmin inhibition in vivo[8] led us to investigate whether plasmin may contribute to the pathogenesis of cartilage degradation and invasion by the pannus tissue in RA.

Invasion into the cartilage can not occur without degradation of the cartilage matrix. To study synovial fibroblast-dependent cartilage degradation, an in vitro model using a radiolabeled cartilage-like matrix was used. We observed that the serine protease inhibitor BPTI completely blocked synovial fibroblast-dependent cartilage matrix degradation. Anti-uPA, but not anti-tPA inhibited degradation significantly, suggesting that plasmin mediates synovial fibroblast-dependent cartilage matrix degradation and that uPA is responsible for plasminogen activation. Blocking plasminogen activation with anti-uPA did not completely inhibit cartilage matrix degradation, suggesting that serine proteases other than plasmin may also be involved. BPTI is a potent inhibitor of plasmin, but also of other serine proteases such as neutrophil elastase[23] and in particular Cathepsin G[24] which is also produced by

fibroblasts.[25] These proteases are also implicated in joint destruction in RA.[26] Plasmin is a broad-spectrum protease that is capable of degrading a wide variety of extracellular matrix proteins directly or indirectly through the activation of latent MMPs.[4] Proteoglycans can be directly degraded by plasmin;[27] degradation of crosslinked collagen most likely occurs through the activation of latent MMPs[28] that are also secreted by the fibroblasts of the pannus tissue.

In the present study, we have investigated a novel strategy to inhibit plasmin at the cell surface of synovial fibroblasts by adenoviral transfer of a gene encoding a cell surface-binding plasmin inhibitor. This inhibitor consists of a plasmin inhibitor (BPTI) linked to the receptor-binding aminoterminal fragment of uPA (ATF). First, we investigated the effects of ATF.BPTI gene transfer on synovial fibroblast-dependent cartilage degradation *in vitro* and compared the effects with those of gene transfer of BPTI or ATF only. A 76% reduction of cartilage matrix degradation was observed after ATF.BPTI gene transfer. The effect of ATF.BPTI gene transfer was significantly stronger than that of BPTI gene transfer. ATF gene transfer did not affect synovial fibroblast-dependent cartilage matrix degradation at all, despite ATF levels at least as high as the ATF.BPTI levels measured after gene transfer. This suggests that binding of uPA to uPAR is of minor importance for plasminogen activation in this model. In cancer invasion in particular, binding of uPA to uPAR appears to be essential;[9] in other processes involving migration of cells, uPAR is not essential.[29]

In our in vitro model, binding of plasmin to the cell surface appears to be important. Targeting plasmin inhibition to the cell surface, as achieved by ATF.BPTI, inhibits cartilage matrix degradation more effectively than does the nonbinding inhibitor BPTI. The finding that blocking binding of ATF.BPTI to the receptor by an antibody against uPAR diminishes the inhibitory effect toward the level observed after BPTI gene transfer underlines the relevance of targeting plasmin inhibition to the cell surface. Furthermore, local expression per se appears to contribute to the efficiency of ATF.BPTI gene transfer as well, since local production of 0.2 KIU/ml BPTI produces the same effect as the addition of 1 KIU/ml exogenous BPTI. These results indicate that both cell surface-targeting and local production contribute to the efficiency of ATF.BPTI gene transfer to inhibit synovial fibroblast-dependent proteolysis.

The *in vitro* results support the hypothesis that plasmin is involved in cartilage degradation by the synovial fibroblasts of the pannus tissue. Cell surface-targeted plasmin inhibition by ATF.BPTI gene transfer provides an effective tool to inhibit synovial fibroblast-mediated cartilage matrix degradation. One can envision that, by inhibiting cartilage degradation, the invasive growth of the pannus tissue into the cartilage is hampered.

To investigate the effects of ATF.BPTI gene transfer on cartilage invasion by RA synovial fibroblasts, the SCID mouse coimplantation model was used. In this model, cartilage invasion is studied by coimplanting RA synovial fibroblasts with human articular cartilage under the renal capsule of an immunodeficient mouse. In comparison with synovial fibroblasts transduced by the control vector, a significant reduction of the invasive growth into the

cartilage was observed. Both the control and the ATF.BPTI-transduced fibroblasts showed migration towards the edge of the cartilage, suggesting that the inhibitory effect of ATF.BPTI gene transfer on invasion is not caused by impaired migration of the cells, but was most likely caused by impaired proteolytic degradation of the cartilage. Invasion was not completely blocked by ATF.BPTI gene transfer. This may have been due to insufficient levels of ATF.BPTI throughout the experiment or to the involvement of other proteolytic enzymes in cartilage invasion.

The findings presented here indicate that cell surface-targeted plasmin inhibition by ATF.BPTI gene transfer may provide a therapeutic tool for inhibiting cartilage destruction at the invasive front of the pannus tissue in RA. However, a possible adverse effect of intra-articular plasmin inhibition in RA may be the interference in the lysis of fibrin depositions, a common phenomenon in RA. Recent work investigating arthritis in uPA and plasminogen knockout mice suggests that impaired fibrinolysis within the joints indeed contributes to excessive inflammation.[30] On the one hand, inhibition of plasmin may inhibit cartilage destruction. On the other hand, it may enhance inflammation by inhibiting fibrinolysis in the joint cavity. Targeting the plasmin inhibitor to the cell surface of invading cells of the pannus may provide a tool to preferentially inhibit cartilage destruction without interfering in fibrin dissolution elsewhere in the joint. To rule out adverse effects such as interfering in fibrin dissolution and to further investigate the effects of ATF.BPTI gene transfer in arthritis, studies in animal models of arthritis should be carried out.

This study provides *in vitro* and *in vivo* evidence for the involvement of the plasminogen activation system in the degradation and invasion of articular cartilage by synovial fibroblasts of the pannus tissue in RA, and demonstrates an effective way to inhibit the pannus-related cartilage destruction by gene transfer of a cell surface-targeted plasmin inhibitor.

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5

NO THERAPEUTIC EFFECT OF PLASMIN ANTAGONIST TRANEXAMIC ACID IN RHEUMATOID ARTHRITIS. A DOUBLE-BLIND PLACEBO-CONTROLLED PILOT STUDY

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Submitted

Summary

Plasmin is one of the enzymes that are implicated in the pathogenesis of joint destruction in rheumatoid arthritis (RA). In the present study, the effects of tranexamic acid, an inhibitor of plasminogen activation, on urinary pyridinoline excretion rates were investigated in RA patients.

The study was set up as a double-blind placebo-controlled pilot study. 19 RA patients with moderate disease activity were included. 10 patients received tranexamic acid and 9 patients received placebo for 12 weeks. Patient assessments were performed before the treatment was administered, at 4, 8, and 12 weeks of treatment, and finally at 4 weeks after cessation of the treatment. Urinary excretion rates of hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) were used as molecular markers of articular cartilage and bone destruction and were measured at every visit. In addition, clinical parameters of disease activity were assessed and CRP levels were measured.

Treatment with tranexamic acid did not result in any effect on pyridinoline excretion rates as compared with placebo, nor was any effect observed on clinical parameters of disease activity or on CRP levels.

In conclusion, the results of the present pilot study indicate that systemic treatment with an inhibitor of plasmin, such as tranexamic acid, has no beneficial effect as adjuvant therapy with respect to inhibition of joint destruction in RA patients with mild or moderate disease.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease involving multiple joints, leading in most cases to irreversible destruction of the articular cartilage and bone. Particularly in the later stages of the disease, the extent of joint destruction largely determines the functional capacity of RA patients.[1] Arresting the progression of joint destruction is therefore an important target in the treatment of RA.

Proteolytic enzymes secreted at the site of destruction cause degradation of the articular cartilage and bone. Although all classes of proteolytic enzymes seem to be involved, cartilage destruction has mainly been attributed to matrix metalloproteinases (MMPs) and serine proteases.[2] Among the serine proteases, plasmin and plasminogen activators may be of interest, because of their fibrinolytic action, their ability to degrade a wide variety of extracellular matrix proteins and to activate latent forms of MMPs.[3] Increased expression of urokinase-type plasminogen activator (uPA) and its receptor (uPAR) in synovial tissues of RA patients,[4,5] as well as the capacity of plasmin to degrade cartilage[6,7] and a bone-like matrix[8] supportthe idea that the the PA-system plays a role in the pathogenesis of joint destruction in RA.

Tranexamic acid is a plasmin antagonist that is currently used in patients as an antifibrinolytic agent. Tranexamic acid is a widely used drug that has only mild adverse effects. After oral administration, tranexamic acid is distributed over the extracellular and intracellular compartments and diffuses rapidly into the synovial fluid.[9,10] In experimental settings, tranexamic acid has appeared to be an inhibitor of cartilage and bone degradation *in vitro*[8] and *in vivo* in arthritis conditions. Beneficial effects of tranexamic acid have been demonstrated in animal models of osteoarthritis[11,12] and in adjuvant arthritis in rats.[13] In adjuvant arthritis, treatment with tranexamic acid reduced the excretion rates of hydroxylysylpyrdinoline (HP), a collagen cross-link that is excreted in urine as a breakdown product of bone and cartilage, indicating a decreased degradation of these tissues in the affected joints. In an open study with 10 RA patients a reduction of excretion rates of both HP and the bone-specific collagen cross-link lysylpyridinoline (LP) was observed after 12 weeks of treatment with tranexamic acid. The suppressive effect disappeared after discontinuation of the treatment.[13] This suggests that inhibition of the PA-system may slow down the progression of bone and cartilage degradation in rheumatoid arthritis.

To further investigate the effects of tranexamic acid, a double-blind placebo-controlled study was performed. The aims of this study were to determine the effects of 12 weeks of tranexamic acid treatment, as adjuvant medication in patients with rheumatoid arthritis, on cartilage and bone degradation as assessed by urinary HP and LP excretion rates.

Patients and Methods

Patients

Nineteen patients from the outpatient clinic of Leyenburg Hospital in The Hague, The Netherlands, with active RA according to the 1987 criteria of the American Rheumatism Association[14] were included after informed consent was obtained. Further inclusion criteria were: age of ≥16 years; stable second-line therapy; and a daily dose of corticosteroids of less than 7.5 mg. Patients with a Steinbrocker stage of IV, renal impairment, with a history of thrombo-embolic disease, and patients who were pregnant or planning to become pregnant were excluded from the trial.

Study design

The local institutional ethics committees approved the study protocol. The study was set up as a double-blind, placebo-controlled pilot study. Patients were treated with either tranexamic acid (Cyklokapron®, Pharmacia Upjohn BV, The Netherlands; 1.5 mg 3 times a day) or a placebo for 12 weeks. The patients visited the outpatient clinics on 7 occasions: 2 weeks and 1 week before the start of the study, on the first day of the study period, at 4, 8, and 12 weeks during the study period, and at 4 weeks after the study period. No alterations in DMARD medication and no intra-articular injections were allowed during the trial. Concomitant therapies are listed in table 1. Tranexamic acid and placebo were prepared in identical capsules and were added to the patients' therapy. We checked the compliance by pill counting. Patients were interviewed about the appearance of new symptoms and side effects that they attributed to the study medication.

Clinical assessments

At inclusion the following demographic and disease characteristics were noted: sex, age, the presence of erosions, the presence of a positive IgM rheumatoid factor (RF; defined as >30 U/ml), and the use of NSAIDs, DMARDs or corticosteroids. At each visit, the following clinical assessments of disease activity were performed: a patient overall assessment of current disease activity on a visual analogue scale (VAS) of 0-100 mm; the duration of morning stiffness (minutes); swollen and tender joint count (28 joints).[15]

Laboratory assessments

At each visit, serum and urine were collected and stored in –80 °C until use. The CRP was measured and urinary pyridinolines HP and LP were measured using HPLC.[16] Normal values for healthy people were assessed in a group of 36 adults. To assess renal toxicity, serum creatinin was measured at each visit.

	Tranexamic acid	Placebo
Males / females: n	1 / 9	1 / 7
Age: mean (SD)	60.8 (13.9)	49.1 (11.0)
Disease duration: y (range)	5.7 (1-30)	9.9 (1-20)
RF positive: n	6	8
Erosions present: n	4*	7
NSAIDs: n	7	6
DMARDs: n	10	8
Methotrexate	5	6
Sulasalazine	3	2
Antimalarials	1	0
Cyclosporin	1	0
Corticosteroids: n	0	2

Table 1. Demographic and disease characteristics of the patients taking tranexamic acid or placebo. *RF = rheumatoid factor; NSAIDs = non-steroidal anti-inflammatory drugs; DMARDs = disease modifying anti-rheumatic drugs. * There were significantly less patients with erosions in the tranexamic acid group than in the placebo group (Pearson Chi-square, p = 0.04)*

Statistical analysis

Pearson Chi-square tests were used to test for differences between the treatment groups in sex, presence of erosions, RF, the usage of NSAIDs, DMARDs or corticosteroids, the number of patients who reported adverse effects, and the number of patients who discontinued the study. A Student's t test was performed to test for differences in age between groups. Mann-Whitney tests were performed to test for differences in disease duration and in baseline values of tender and swollen joint counts, VAS, CRP, DAS, HP, and LP between the treatment groups. The efficacy parameters were analyzed on the basis of an intention-to-treat analysis. For patients who prematurely discontinued the study, the data from the visit at which the trial medication was stopped were carried forward. Differences between courses of all outcome parameters during the treatment period were evaluated by repeated measures analysis of variance. Changes in the disease activity parameters and HP and LP excretion rates during the treatment period were assessed by subtracting the values at baseline from the values at week 4, 8, or 12. Differences in changes of all parameters between the tranexamic acid- and the placebo-treated group were evaluated by Student's t tests. A power analysis was performed based on the baseline HP and LP levels at baseline of the patients who participated in this study. Assuming no changes during the treatment of the HP and LP excretion rates in the placebo group, a

change of 20% of the baseline values in the tranexamic acid group was considered clinically relevant. A power of 0.8 and a significance level of 0.05 were chosen. All statistical calculations, except for the power analysis, were performed using SPSS 10.0 for Windows. P values of less than 0.05 were considered to be statistically significant.

Results

Power analysis revealed that 9 patients per group were required to allow statistical support for a 20% difference in the changes of HP and LP excretion rates between the tranexamic acid and placebo group during the treatment with the study medication. 10 patients received tranexamic acid and 9 patients placebo. 16 patients completed the study. The compliance was good. One patient in the tranexamic acid-treated group discontinued the study medication after 4 weeks because of a peripheral nervus facialis paralysis (Bell's Palsy). Neurological evaluation revealed no thrombo-embolic event. Another patient in the tranexamic acid-treated group was excluded from the study after 4 weeks, because of a history of thrombosis risk that was not previously reported. One patient in the placebo group discontinued the study medication within the first days after the start of study medication because of a panaritium of her finger. She was excluded from the analysis.

Erosions were present in significantly more patients in the placebo group than in patients in the tranexamic acid group. There were no significant differences between the two groups with respect to sex, age, disease duration, presence of RF, or their NSAID or DMARD medication (Table 1).

7 patients in the tranexamic acid-treated group experienced mild diarrhea, throughout the treatment period. The symptoms stopped after cessation of the TEA treatment. None of the patients in the placebo group experienced diarrhea-like symptoms. 1 patient in the placebo group reported an increase in RA symptoms. Nausea was reported by 1 patient in the placebo group. In the tranexamic acid-treated group one patient complained of a headache at week 12 of the treatment.

No effect of tranexamic acid on clinical parameters of disease activity or CRP

The effect of tranexamic acid on the following disease activity parameters was determined at the start, at week 4, 8, and 12 during treatment, and at 4 weeks after discontinuation of the study drug treatment: patient global assessment (VAS); tender and swollen joint count; mDAS; duration of morning stiffness; and CRP. For all outcome parameters at baseline, there were no significant differences between the treatment groups. The CRP levels were low for both groups (Table 2).

Repeated measures analysis of variance showed no significant differences between the treatment groups in the courses of any of the disease activity parameters. The changes in the disease activity parameters during the treatment period did not differ between the tranexamic acid-treated and the placebo-treated groups (Table 2). Analyzing the data of the patient with erosions only, revealed no effect of tranexamic acid on any of disease activity parameters.

	Tranexamic acid (n=10)		Placebo (n=8)	
	Baseline	**12 weeks**	**baseline**	**12 weeks**
Patient assessment (VAS, mm)	57.5 (1-80)	59 (4-86)	51 (24-82)	57. (38-71)
Swollen joints (nr)	3 (0-16)	4 (0-10)	5.5 (0-14)	4 (0-11)
Tender joints (nr)	5 (0-18)	2.5 (0-16)	4 (0-15)	7 (1-19)
Dur. morning stiffness (min)	15 (0-180)	10 (0-180)	22.5 (0-90)	22 (0-90)
CRP (mg/l)	<5 (<5-88)	<5 (<5-31)	11 (<5-24)	12.5 (<5-43)
DAS	2.7 (1.5-6.2)	3.1 (2.0-5.0)	4.1 (0.7-6.2)	4.5 (1.5-6.1)
HP (nmol/mmol creat)	91.3 (60.7-193.8)	92.8 (60.3-150.8)	108.0 (74.6-163.8)	92.2 (54.8-
LP (nmol/mmol creat)	23.0 (15.3-65.7)	25.1 (7.7-39.5)	26.4 (18.3-32.18)	22.1 (8.6-27.1)

Table 2. Values of clinical and laboratory parameters of disease activity and joint destruction at baseline and after 12 weeks of treatment with tranexamic acid or placebo. *Values are median (range). VAS = 100 mm visual analogue scale; CRP = C-reactive protein; DAS = disease activity score; HP = urinary excretion rates of hydrolxylysylpyridinoline; LP = urinary excretion rates of lysyslpyridinoline.*

No effect of tranexamic acid on pyridinoline excretion rates.

There were no significant differences in HP and LP excretion rates at baseline between the tranexamic acid-treated and placebo-treated groups. Normal values in healthy volunteers were <40 nmol/mmol creatinin for HP and <10 nmol/mmol creatinin for LP. The urinary HP and LP excretion rates of the patients participating in the present study were significantly higher than in the healthy control subjects ($p < 0.001$). In all cases the HP and LP excretion rates were above normal values. Repeated measures analysis of variance showed no significant differences between the groups in the courses of the excretion rates of HP and LP during the study period. Analysis of the changes (Δ) of the urinary excretion rates of HP and LP at the start and the end of the 12-week treatment period showed no differences between patients treated with tranexamic acid or placebo (Figure 1). Analyzing the data of the patient with erosions only or the data of patients with the higher HP excretion rates at baseline (> 100 nmol/mmol creatinin) revealed no effect of tranexamic acid on HP or LP excretion rates.

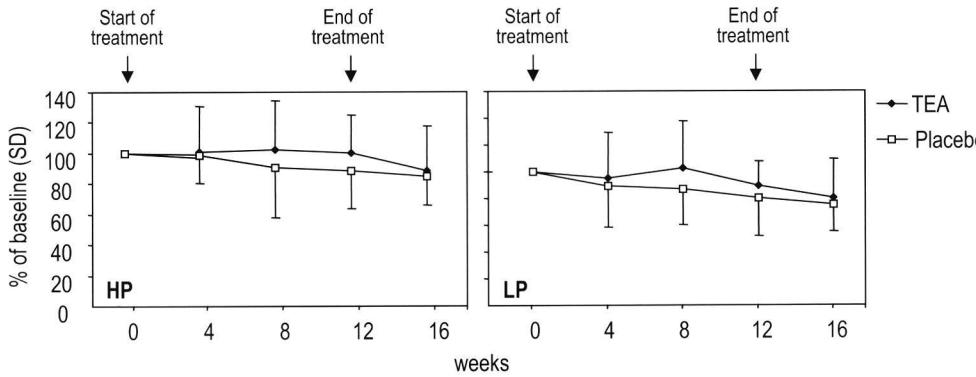

Figure 1. Urinary pyridinoline excretion rates during treatment with tranexamic acid or placebo. *Treatment with tranexamic acid during 12 weeks did not result in a decrease of urinary HP (left panel) or LP excretion rates (right panel) as compared with the placebo.*

Discussion

The aim of the present study was to investigate whether tranexamic acid may be beneficial for RA patients as an additional drug to inhibit the progression of joint destruction. In contrast to previous findings in an open study with RA patients,[13] no effects of tranexamic acid on urinary pyridinoline excretion rates were observed in the present double-blind, placebo-controlled study. No effects of tranexamic acid were observed either on the parameters of disease activity. This was in line with previous findings showing no effects of tranexamic acid on disease activity parameters in RA patients.[17]

The lack of effect could not be explained by low pyridinoline excretion rates at baseline, since in all patients the baseline pyridinoline excretion rates were elevated compared with the control subjects. In the majority of tranexamic acid-treated patients, mild diarrhea was experienced, which is a common adverse effect. Even though the patients reported a good compliance, it cannot be ruled out that some of these patients did not take their study medication properly. If that is the case, this may have contributed to the lack of effect of tranexamic acidobserved in the present study.

The results of this study suggest that systemic inhibition of plasmin is not an effective therapeutic strategy to inhibit joint destruction in RA. Even though previous studies indicate that plasmin is directly involved in cartilage and bone destruction in RA,[4,6,8] other proteinases, including MMPs and cathepsins, are also implicated. Therefore, inhibiting plasmin only may not be sufficient to inhibit joint destruction in RA patients. Possibly, the observed decrease in pyridinoline excretion rates in the open study[13] was caused by factors other than the treatment

with tranexamic acid. However, the observation that the decrease of pyridinoline excretion rates during tranexamic treatment was followed by an increase after cessation of the treatment, strongly suggests that the effect was in fact due to tranexamic acid.

Another explanation may be that treatment with plasmin inhibitors is only effective in RA patients with very active disease. In the open study only patients with very active, erosive disease were included,[13] whereas the disease activity in the patients who participated in the present study was mild or moderate. Moreover, in some of these patients erosions were absent. Since proinflammatory mediators, such as IL-1 and granulocyte macrophage colony stimulating factor increase the expression of uPA,[18] it is conceivable that plasminogen activation is decreased in the synovial tissues of patients with low disease activity compared with those of patients with active disease. Unfortunately, it was not possible to investigate the expression of uPA and other components of the PA-system in the synovial tissues or synovial fluid of these patients, since synovial biopsies or synovial fluid samples were not taken during the study.

Based on the results of the present study it can be concluded, that treatment with tranexamic acid has no beneficial effect as adjuvant therapy with respect to inhibition of joint destruction in patients with mild or moderate RA.

Acknowledgements:

The authors wish to thank Mrs. Ingeborg Henkes and Mrs. Joke Gilles for evaluating the patients and Mr. Nico Sakkee and Mr. Dirk-Jan van der Berg for the HP and LP measurements.

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6

CARTILAGE DEGRADATION AND INVASION BY RHEUMATOID SYNOVIAL FIBROBLASTS IS INHIBITED BY GENE TRANSFER OF TIMP-1 AND TIMP-3.

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Submitted

Summary

Invasion of articular cartilage by the pannus tissue is an important cause of joint destruction in RA. Matrix metalloproteinases (MMPs) are believed to be pivotal enzymes in this process. We investigated the effects of gene transfer of tissue inhibitors of MMPs on the invasive behavior of rheumatoid synovial fibroblasts *in vitro* and *in vivo*.

Adenoviral vectors (Ad) were used for gene transfer. AdLacZ and a vector without an insert were used as control constructs. The effects of AdTIMP-1 and AdTIMP-3 gene transfer on invasion were investigated *in vitro* in a transwell system. Cartilage invasion *in vivo* was investigated after 60 days in the SCID mouse co-implantation model. In addition, the effects of AdTIMP-1 and AdTIMP-3 gene transfer on cell proliferation were investigated.

A significant reduction in invasiveness was demonstrated *in vitro* as well as *in vivo* in both the AdTIMP-1- and AdTIMP-3-transduced rheumatoid synovial fibroblasts compared with the synovial fibroblasts transduced with the control vectors or the untransduced cells. *In vitro*, the number of invading cells was reduced to 25% ($p < 0.001$) in the AdTIMP-1-transduced cells and to 13% ($p < 0.0001$) in the AdTIMP-3 transduced cells, compared with 61% in the AdLacZ-transduced cells (% of untransduced cells). Cell proliferation was significantly inhibited by AdTIMP-3 and, to a lesser extent, by AdTIMP-1 gene transfer.

In conclusion, overexpression of TIMP-1 and TIMP-3 by adenoviral gene transfer results in a marked reduction of the invasiveness of rheumatoid synovial fibroblasts *in vitro* and in the SCID mouse co-implantation model. Apart from the inhibition of MMPs, a reduction in proliferation rate may have contributed to this effect. These results suggest that overexpression of TIMPs, particularly TIMP-3 at the invasive front of the pannus tissue may provide a novel therapeutic strategy for inhibiting joint destruction in RA.

Introduction

The invasion of synovial cells into the articular cartilage and bone is an important cause of joint destruction in RA.[1] Activated synovial fibroblasts have been shown to be involved in this process by their attachment to the cartilage surface and the release of matrix-degrading enzymes.[2] These locally secreted proteolytic enzymes degrade the articular cartilage at the pannus-cartilage interface and mediate invasion of the synovial cells into the cartilage. Although all classes of proteolytic enzymes seem to be involved, it is generally believed that matrix metalloproteinases (MMPs) are pivotal in cartilage destruction.[3,4]

The MMPs are a family of zinc-dependent proteolytic enzymes that have the ability to degrade almost all proteins of the extracellular matrix. MMPs are divided in subgroups including: collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -7,-10,-11,-12), and membrane-type (MT) MMPs (MT1-MMP, MT2-MMP, MT3-MMP, MT4-MMP also termed MMP-14,-15, -16, and -17). MMPs are secreted as proenzymes and activated by serine proteases like plasmin or elastase, or by other MMPs including MMP-3 and MT1-MMP. In rheumatoid synovial tissue, increased amounts of MMPs are expressed.[5,6-10] MMP levels in the synovium and serum correlate with disease activity[11] and radiographic damage,[12,13] suggesting that MMPs may play a role in the destructive process in RA. Therefore, inhibition of MMPs may be a therapeutic strategy to prevent joint destruction in RA, and the delivery of naturally occurring inhibitors of MMPs may serve as one option for achieving this goal.

Within the tissues, MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs). Four subtypes of TIMPs have been described,[14] that share some properties, but are distinct in others. TIMP-1, TIMP-2, and TIMP-4 are secreted as soluble factors, while TIMP-3 is associated with the extracellular matrix. Unlike TIMP-1, TIMP-2 and TIMP-3 are effective inhibitors of the MT-MMPs. Moreover, in contrast to the other TIMPs, TIMP-3 is capable of inhibiting the shedding of cell membrane-anchored proteins such as tumor necrosis factor (TNF) receptor,[15] interleukin-6 receptor,[16] syndecan ectodomains[17] and of inhibiting TNF-α-converting enzyme (TACE).

The presence of TIMPs has been shown in RA synovial tissue.[5,21] However, despite an increased expression of TIMP in RA,[5,22] proteolytic degradation of the articular cartilage still occurs, suggesting an imbalance between MMP and TIMP activity in favor of the MMPs. Restoring the imbalance between MMP and TIMP activity within the rheumatoid synovium or changing it into a balance in favor of TIMP activity may protect the articular cartilage and bone from MMP-mediated degradation.

In the present study, we investigated the effects of overexpression of TIMP-1 or TIMP-3 by adenoviral (Ad) gene transfer on the invasive behavior of rheumatoid synovial fibroblasts *in vitro* and in the SCID-mouse co-implantation model.

Material and Methods

Adenoviral vectors.

Replication-defective adenoviral vectors (E1-deleted, CMV promotor) encoding TIMP-1 and TIMP-3 were used for the experiments. As control vectors, a β-galactosidase-encoding adenoviral construct (AdLacZ) and a construct without an insert (AdControl) were used.

Isolation and culture of rheumatoid synovial fibroblasts.

Synovial tissues were obtained with informed consent from RA patients who required joint surgery. RA synovial fibroblasts were isolated by collagenase digestion and cultured as described previously.[4] The rheumatoid synovial fibroblasts were passaged two or three times before use in the *in vitro* experiments. For the SCID mouse co-implantation experiment, rheumatoid synovial fibroblasts passaged four times were used. The rheumatoid synovial fibroblasts that were implanted in the SCID mice (untransduced cells, cells transduced with AdTIMP-1, AdTIMP-3, or AdControl) were also cultured for 60 days in DMEM/F12 supplemented with 10% fetal calf serum (FCS) without passaging the cells.

Transduction: *In vitro experiments*: Rheumatoid synovial fibroblasts were seeded in 6-well plates (2×10^5 cells per well) in DMEM/F12 medium with 10% normal human serum (NHS) and 10% newborn calf serum (NCS). After 24 hours, the cells were washed and incubated with an adenoviral vector in concentrations varying from 30 to 1000 plaque-forming units (PFU) per cell in 1 ml DMEM/F12 supplemented with 5% NCS or 5% FCS. After overnight incubation, the adenovirus-containing medium was removed.

SCID mouse co-implantation model: 5×10^5 rheumatoid synovial fibroblasts were seeded in 75 cm^2 culture flasks and were incubated for 24 hours with 300 PFU/cell of AdTIMP-1, AdTIMP-3, or the mock vector in 15 ml DMEM/F12 with 5% FCS. After 24 hours, the cells were washed and incubated for 24 hours with DMEM/F12 with 10% FCS. Transduction efficiency was assessed in the LacZ -ransduced fibroblasts after β-galactosidase staining by assessing the percentage of blue-stained cells by cell counting.

mRNA isolation and reversed transcriptase (RT) PCR.

Rheumatoid synovial fibroblasts, transduced with AdTIMP-1, AdTIMP-3, AdControl, or untransduced cells were cultured after transduction in DMEM/F12 supplemented with 10% NHS and 10% NCS. After two days of culture, mRNA was isolated using the Chomczynski procedure.[23] The rheumatoid synovial fibroblasts (transduced with AdTIMP-1, AdTIMP-3, AdControl, or untransduced cells) that were cultured in parallel to the SCID mouse co-implantation experiment, were lysed after 60 days of culture. Of these lysates, mRNA was isolated using the TRIZOL®LS Reagent (GibcoBRL, Life Technologies AG, Basel, Switzerland). Of the mRNA isolated, 1 µl was used to generate cDNA using the Reverse Transcription System of Promega (Leiden, The Netherlands). cDNA was amplified by PCR (RoboCycler, Stratagene Europe, Amsterdam, The Netherlands). To specifically detect

mRNA of the transgenic TIMP-1 and TIMP-3, primer pairs were designed that recognized TIMP-1 or TIMP-3 in combination with mRNA of the adenoviral construct that is expressed along with the TIMP-1 or TIMP-3 genes. To detect transgenic TIMP-1, oligonucleotide 5'-TCGCGATGCACCTGTGTCCCACC-3', recognizing TIMP-1 cDNA, was used as the forward primer (TIMP-1 forw). To detect transgenic TIMP-3, oligonucleotide 5'-AGCAGCGGCAATGACCCCTTG-3', recognizing TIMP-3 cDNA was used as the forward primer (TIMP-3 forw). In both cases oligonucleotide 5'-TCTAGCAGCACGCCATAGTGAC-3', recognizing the DNA of the plasmid that was inserted into the adenoviral vectors, was used as the reversed primer (Adrev).

Cell viability assessment.

Rheumatoid synovial fibroblasts were seeded in a 24-well plate (2.5×10^4 cells/well) and incubated with AdTIMP-1, AdTIMP-3, AdLacZ, or AdControl in concentrations of 100, 200, 500 or 1000 PFU/ml. After overnight incubation, the cells were washed and cultured in serum-free DMEM/F12. After 24 hours, the cells were washed and incubated for 3 hours in 37°C with an MTT solution (1 mg MTT/ml PBS; Sigma Chemical Company, St Louis, MO). After 3 hours the cells were lysed. Of the lysates 100 µl was transferred to an ELISA-plate and extinction was measured at 540 nm. The results are expressed as means and standard error of the mean (SEM).

Proliferation assay.

Incorporation of ^{3}H-thymidine in DNA was determined to measure cell proliferation. RA synovial fibroblasts were seeded at 50% confluency (2.5×10^4 cells/well) in 24-well tissue culture plates in DMEM/Ham's F12 supplemented with 5% NCS. After 24 hours, the cells were incubated with AdTIMP-1, AdTIMP-3, or AdLacZ in concentrations of 100, 200, 500 or 1000 PFU/ml. After overnight incubation, the medium was changed, and the cells were incubated with DMEM/F12 supplemented with 40% NHS for 26 hours at 37°C and labeled with 1µCi ^{3}H-thymidine/well (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) for the last 4 hours. ^{3}H-thymidine incorporation was measured as described previously.[24] The results are expressed as means and SEM.

TIMP-1 measurement.

To assess the rate of overexpression of TIMP-1 and the duration of gene expression after gene transfer rheumatoid synovial fibroblasts were cultured after transduction (MOI = 300) with AdTIMP-1 or AdControl for 60 days (5×10^5 cells/ 75 cm^2). Every ten days supernatants were taken and stored at -80°C until measurement. TIMP-1 was measured in the supernatants by ELISA (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). The results are expressed as means and SEM.

MMP activity assay.

To investigate whether transduction with AdTIMP-1 or AdTIMP-3 leads to overexpression of active TIMP-1 and TIMP-3 (capable of inhibiting MMP activity) an MMP activity assay was used. Cell lysates and conditioned medium samples (24 hours, serum-free) were incubated with active MMP-2 (final concentrations of 0.5 nM) at 37°C for 120 minutes. Dilutions of the cell lysates (10 ×) and conditioned media (200 ×) were chosen that showed no inhibitory activity in the samples of the untransduced (control) and AdLacZ-transduced fibroblasts, but by contrast a clear inhibitory activity in the samples of AdTIMP-1- and/or AdTIMP-3-transduced fibroblasts. Then, a fluorescent MMP-2-sensitive substrate (TNO 211F, TNO Prevention and Health, Gaubius Laboratory, Leiden, The Netherlands) was added, and directly thereafter MMP activity was measured in a fluorescence multiplate reader (Cytofluor II, Applied Biosystems, Foster City, CA) every 3 minutes over a 90 minute period. The MMP activity was assessed within the time interval of linear increase of MMP activity over time and was defined as fluorescence units per second (FU/s).

Gelatin zymography.

To investigate whether transduction with AdTIMP-1 or AdTIMP-3 leads to inhibition of the activation of pro-MMP-2, gelatine zymography was performed. Using this technique the presence of active MMP-2 can be distinguished from latent MMP-2. Rheumatoid synovial fibroblasts were seeded on top of Matrigel® (Collaborative Biochemical Products, Bedford, MA) or collagen I (Vitrogen 100, Celtrix, Santa Clara, CA, USA). The fibroblasts were transduced with AdTIMP-1, AdTIMP-3, or the mock vector as described above. The fibroblasts were cultured for two days in DMEM/F12 supplemented with 10% NHS and 10% NCS in the presence of 5 ng/ml TNF-α to stimulate MT1-MMP expression. The medium was washed away and the cells were cultured for two additional days in serum-free DMEM/F12 with 5 ng/ml TNF-α. Then the conditioned medium samples were collected and gelatinolytic activity was assessed by applying the samples to a 10% (w/v) acrylamide gel containing 0.2% (w/v) gelatin as described previously.[25]

***In vitro* invasion model.**

Invasion of rheumatoid synovial fibroblasts *in vitro* was assessed using a Transwell system with polycarbonate filters (6.5 mm diameter, 8 μm pore size; Costar, Cambridge, MA). To achieve a barrier for the cells to invade through, Matrigel® or collagen type I was used. Matrigel® was layered on top of the filters in DMEM/F12 (37.5 μg/well) and dried overnight at room temperature. The Matrigel®-coated filters were hydrated by DMEM/F12 before use. Rheumatoid synovial fibroblasts were seeded in 200 μl serum-free DMEM/ F12 on top of the Matrigel® in the upper chamber of the transwell (2×10^4 cells/well). When collagen I was used as a barrier, rheumatoid synovial fibroblasts were suspended in a collagen I solution (1.9 mg/ml) on ice. Subsequently, the collagen gelled by incubating the collagen I at 37°C. The lower chambers were filled with 900 μl DMEM/F12 supplemented with 10% NHS and 10%

NBCS. After 72 hours of incubation at 37°C in 5% CO_2 / 95% air (v/v) the cells were fixed with 2.5% (v/v) glutaraldehyde and stained with a crystal violet solution (2g/L). The Matrigel® or collagen I together with the cells on the upper side of the filter were scraped off with cotton swabs. Invasion was determined by the % surface area covered by cells as measured by image analysis using an Olympus CK2 inverted microscope equipped with a monochrome CCD camera (MX5). Data were analyzed using Optimas image analysis software (BioScan Inc., Washington DC). To investigate inhibitory effects, MMP inhibitor Marimastat (kind gift from Mrs E. Bone of British Biotech, Oxford, United Kindom) was added in the upper and lower compartments of the transwells in concentrations of 10, 20, 50, or 100 µg/ml. Alternatively, the cells were transduced with AdTIMP-1, AdTIMP-3 or a control vector before seeding onto the Matrigel® or in the collagen I matrix. Rheumatoid synovial fibroblasts (3rd passage) from 5 donors were used. 300 PFU/cell of adenoviral constructs were used in the invasion experiments. All experiments were performed in triplicate. The results are expressed as means and SEM.

SCID-mouse co-implantation model.

At 48 hours after transduction with AdTIMP-1, AdTIMP-3, or a control vector (300 PFU/cell), rheumatoid synovial fibroblasts were trypsinized and resuspended in 250 µl DMEM/F12 with 10% FCS and inserted into 2 mm^3 sterile Gelfoam sponges (Pharmacia & Upjohn, Kalamazoo, MI, USA) as described previously.[2] Fresh articular cartilage of the knee was obtained from the pathology department and cut in 2-3 mm^3 pieces. Under sterile conditions, the sponges containing the transduced fibroblasts were co-implanted with the cartilage pieces under the renal capsule in the SCID mice. A total of twelve mice were used for the implantations: three mice were implanted with AdTIMP-1-transduced fibroblasts, three mice were implanted with AdTIMP-3-transduced fibroblasts, three mice received fibroblasts infected with the control vector, and three mice received non-transduced fibroblasts. After 60 days, the implants were removed and paraffin sections were made. The invasion was scores as decribed previously.[26] Of every co-implant, the invasion scores were determined by taking the means of the sections with the highest score. Per group the means ± SEM of these values were calculated.

Results

Transduction efficiency of AdLacZ-transduced rheumatoid synovial fibroblasts.

At 24 hours after transduction with 300 PFU/cell a transduction efficiency of 67% was achieved, as assessed by counting blue-stained cells.

TIMP-1 and TIMP-3 expression in AdTIMP-1- and AdTIMP-3-transduced cells.

Transgenic TIMP-1 and TIMP-3 mRNA expression was demonstrated at 2 days (data not shown) and 60 days after gene transfer as assessed by RT-PCR (Figure 1). TIMP-1 levels in

the supernatants of AdTIMP-1-transduced fibroblasts were measured by ELISA. In the conditioned medium of the AdTIMP-1- transduced cells TIMP-1 levels were 57.0 µg/ml at 10 days after transduction and 39.0 µg/ml at 60 days after transduction. TIMP-1 concentrations in the conditioned medium of AdControl-transduced cells were 5.8 µg/ml at 10 days after transduction and 0.1 µg/ml at 60 days after transduction.

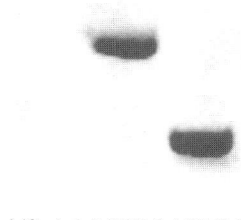

Figure 1. Transgenic TIMP-1 and TIMP-3 expression at 60 days after transduction. *Rheumatoid synovial fibroblasts were cultured after transduction with AdTIMP-1, AdTIMP-3, or the AdControl. At 60 days after transduction, mRNA was isolated of which cDNA was made using reversed transcriptase. The cDNA was amplified by PCR using primer pairs (TIMP-1 forw/Adrev and TIMP-3 forw/Adrev) that reacted specifically with the transgenic TIMP-1 or TIMP-3 and not with the endogenous TIMP-1 and TIMP-3.*

MMP-inhibitory activity after AdTIMP-1 and AdTIMP-3 gene transfer.

Elevated levels of MMP inhibitory activity were measured in the cell lysates of AdTIMP-1 and AdTIMP-3 transduced cells. The inhibitory activity in cell lysates of AdTIMP-1-transduced cells was stronger than in those of AdTIMP-3-transduced cells (Figure 2, white bars). In the conditioned medium of AdTIMP-1-transduced cells MMP-inhibitory activity was found, whereas in the conditioned medium of AdTIMP-3-transduced cells only little MMP-inhibitory activity was found (Figure 2, black bars).

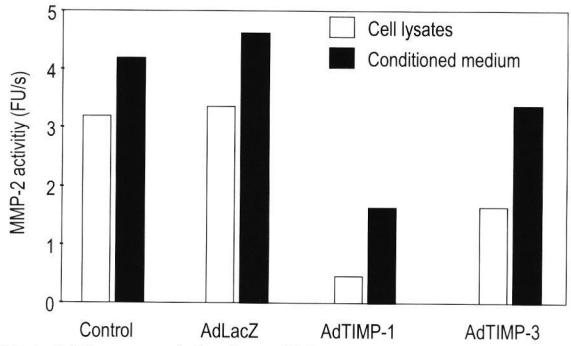

Figure 2. MMP inhibitory activity in cell lysates and conditioned medium of AdTIMP-1- and AdTIMP-3-transduced rheumatoid synovial fibroblasts. *Cell lysates and conditioned medium samples of AdTIMP-1- and AdTIMP-3-transduced rheumatoid synovial fibroblasts were diluted 10 and 200 times respectively and incubated with active MMP-2 for 120 minutes. Subsequently a fluorogenic substrate for MMP-2 was added, and MMP-activity was measured over time.*

To investigate the effects of AdTIMP-1 and AdTIMP-3 gene transfer on MMP-2 activation, gelatine zymography was performed. In the conditioned medium of the untransduced, AdLacZ, and TIMP-1-transduced fibroblasts both active MMP-2 and pro-MMP-2 was present, whereas in the conditioned medium of AdTIMP-3-transduced fibroblasts pro-MMP-2 was present, but hardly any active MMP-2 (Figure 3).

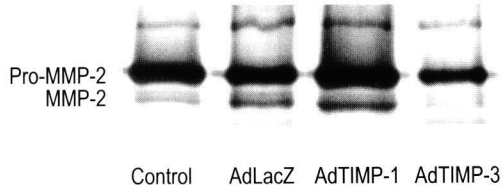

Figure 3. TIMP-3 overexpression inhibits MMP-2 activation. *Rheumatoid synovial fibroblasts transduced with AdLacZ, AdTIMP-1, AdTIMP-3, or untransduced cells (control) were cultured in serum-free medium for 2 days in the presence of 5 ng/ml TNF-α to stimulate MT1-MMP expression. Gelatin zymography was performed in the conditioned medium to investigate the presence of active MMP-2.*

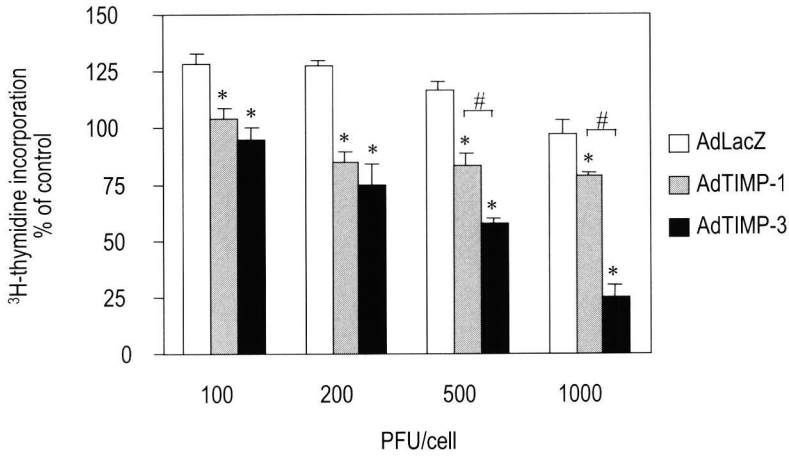

Figure 4. Inhibition of cell proliferation by TIMP-1 and TIMP-3 gene transfer. *De effects transduction with AdTIMP-1 and AdTIMP-3 gene transfer using concentrations of 100, 200, 500, and 1000 PFU/cell on proliferation of rheumatoid synovial fibroblasts was investigated by assessment of the rate of ^{3}H-thymidine incorporation. The ^{3}H-thymidine incorporation in untransduced cells (control) was set as 100%. * = $p < 0.01$; # = $p < 0.05$.*

Effects of AdTIMP-1 and AdTIMP-3 transduction on cell viability.

Cell viability after transduction with AdTIMP-1, AdTIMP-3, or AdLacZ was assessed by measuring mitochondrial enzyme activity. When up to 500 PFU/cell of adenoviral construct were used, no significant reduction was observed on cell viability of AdTIMP-1- and AdTIMP-3-transduced rheumatoid synovial fibroblasts compared with AdLacZ-transduced fibroblasts. When 1000 PFU/cell were used, a 12% reduction of mitochondrial enzyme activity in AdTIMP-1-transduced cells ($p = 0.02$) and a 25% reduction in AdTIMP-3-transduced cells ($p = 0.007$) was measured, as compared with AdLacZ-transduced cells.

Effects of AdTIMP-1 and AdTIMP-3 gene transfer on proliferation.

The proliferation rate of rheumatoid synovial fibroblasts transduced with AdTIMP-1 or AdTIMP-3 was significantly reduced compared with AdLacZ-transduced rheumatoid synovial fibroblasts, as measured by ^{3}H-thymidine incorporation. The effect of AdTIMP-3 gene transfer was significantly stronger than that of AdTIMP-1 gene transfer (Figure 4).

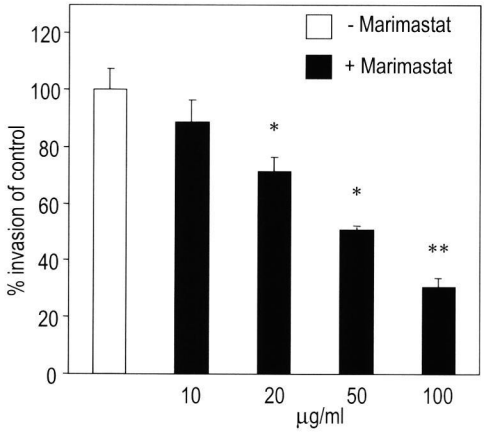

Figure 5. Effects of MMP inhibitor Marimastat on* in vitro *invasion. *The involvement of MMPs in invasion through a Matrigel matrix by rheumatoid synovial fibroblasts was investigated* in vitro *by adding various concentrations of Marimastat.* * = $p < 0.05$; ** = $p < 0.01$.

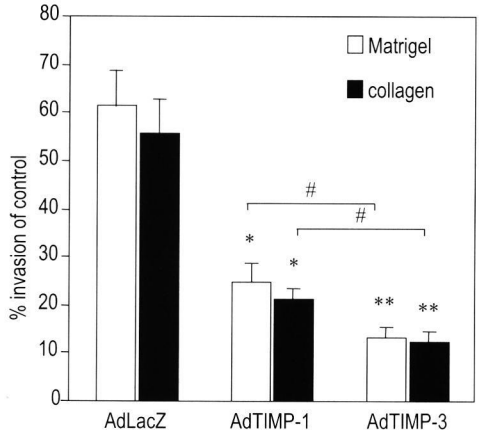

Figure 6. Effects of AdTIMP-1 and AdTIMP-3 gene transfer on* in vitro *invasion. *The effects of AdTIMP-1 and AdTIMP-3 on invasion through a Matrigel or collagen I matrix by rheumatoid synovial fibroblasts was investigated in* in vitro. *AdLAcZ was used as a control adenoviral construct. The invasion is defined as the percentage of invaded cells compared with untransduced rheumatoid synovial fibroblasts.* * = $p < 0.001$; ** = $p < 0.0001$; # = $p < 0.05$.

Inhibition of *in vitro* invasion by MMP inhibitor Marimastat and transduction with AdTIMP-1 and AdTIMP-3.

The invasiveness of rheumatoid synovial fibroblast through a matrix of Matrigel® was significantly inhibited by Marimastat in a dose-dependent way (Figure 5). Transduction of the cells with AdTIMP-1 or AdTIMP-3 resulted in a significant inhibition of invasion through a matrix of Matrigel® or collagen I, as compared with AdLacZ. The number of invading cells reduced to 25% ($p < 0.001$) in the AdTIMP-1-transduced cells and to 13% ($p < 0.0001$) in the AdTIMP-3-transduced cells, compared with 61% in the AdLacZ-transduced cells (% of untransduced cells). The inhibitory effect of AdTIMP-3 gene transfer was significantly stronger than that of AdTIMP-1 (Figure 6).

Inhibition of invasion by transduction with AdTIMP-1 and AdTIMP-3 in the SCID mouse co-implantation model.

Rheumatoid synovial fibroblasts (untransduced, transduced with AdTIMP-1, AdTIMP-3, or AdControl) were co-implanted with human articular cartilage in twelve mice (three per group). The results of three mice could not be evaluated: one mouse which had received AdTIMP-1-transduced fibroblasts did not survive the operation, and in two mice with AdControl-transduced fibroblasts, the cartilage or the sponge became dislocated.

Deep invasion into the cartilage was observed in the untransduced and AdControl-transduced fibroblasts (Figures 7A and 7B, respectively; Figure 8). A clear inhibition of invasion was observed in both the AdTIMP-1- and the AdTIMP-3-transduced fibroblasts (Figures 7C and 7D, respectively; Figure 8). In only a few areas of the cartilage co-implanted with the AdTIMP-1- and AdTIMP-3-transduced rheumatoid synovial fibroblasts, invasion was observed. In contrast to untransduced fibroblasts and AdControl-transduced fibroblasts, these invasive spots were not spread throughout the cartilage and the invasion was less deep (Figures 8E and 8F). Comparison of invasion scores of AdTIMP-3-transduced cells ($n = 3$) with untransduced cells ($n = 3$) revealed a statistical significant reduction of invasion in AdTIMP-3-transduced cells ($p < 0.01$). The difference between the invasion scores of AdTIMP-1-transduced ($n = 2$) and untransduced cells ($n = 3$) did not reach statistical significance ($p = 0.1$).

Discussion

Invasion of the articular cartilage and bone by the pannus tissue leads to destruction of the joints in patients with RA. The involvement of MMPs in this process and the effects of gene transfer of TIMPs were investigated in the present study.

In an in vitro model of invasion, the invasiveness of rheumatoid synovial fibroblast was significantly reduced by Marimastat, indicating that MMPs directly mediate this process. These findings are in line with the concept that the imbalance in the expression of MMPs and TIMPs by rheumatoid synovial cells at the invasive front of the pannus tissue contributes to

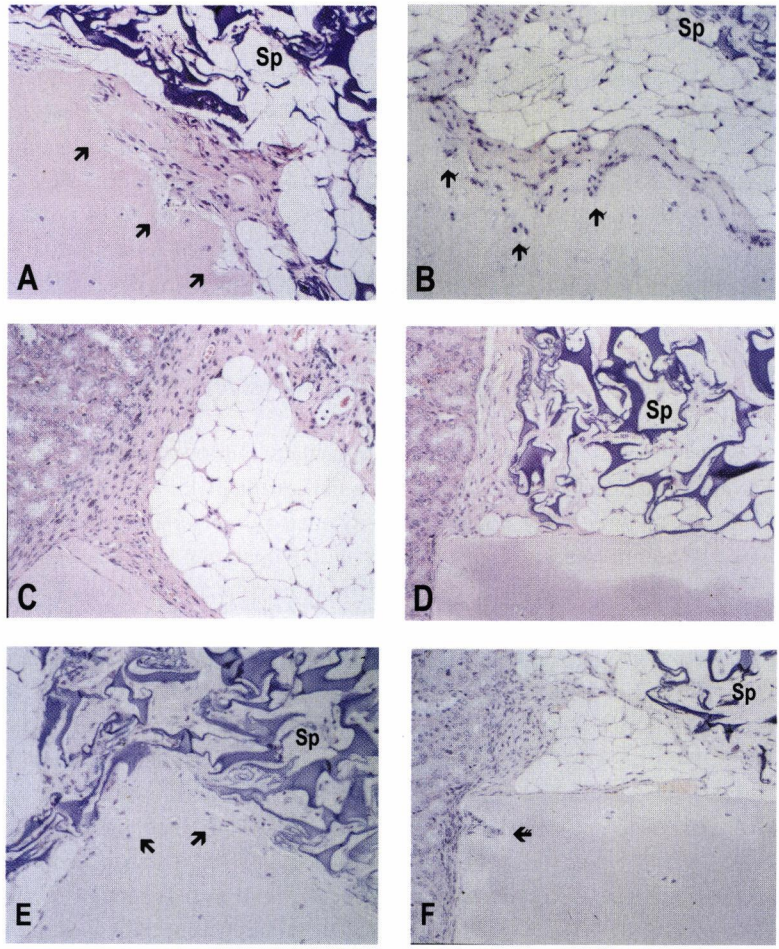

Figure 7. Cartilage invasion by rheumatoid synovial fibroblasts is inhibited by TIMP-1 and TIMP-3 gene transfer in the SCID co-implantation mouse model. *Rheumatoid synovial fibroblasts, transduced with AdTIMP-1, AdTIMP-3, a control vector, or untransduced cells were put into a Gelfoam sponge (Sp) and co-implanted with human articular cartilage under the renal capsule of SCID for a period of 60 days. After this period, the co-implants were removed, paraffin sections were made, stained with haematoxilin and eosin, and the depth of invasion of the rheumatoid synovial fibroblasts was evaluated using a semiquantitive scoring method. The arrows indicate the invasive front of the implanted rheumatoid synovial fibroblasts.* **A***: untransduced rheumatoid synovial fibroblasts;* **B***: AdControl transduced rheumatoid synovial fibroblasts;* **C** *and* **E***: AdTIMP-1 transduced rheumatoid synovial fibroblasts;* **D** *and* **F***: AdTIMP-3 transduced rheumatoid synovial fibroblasts.*

cartilage destruction in RA.[22,27] Since gene transfer of TIMPs may be a way to restore the balance between the expression of MMPs and TIMPs by rheumatoid synovial fibroblasts, the effects of gene transfer of AdTIMP-1 and AdTIMP-3 on the invasiveness of rheumatoid synovial fibroblasts were investigated. First, the inhibitory properties of TIMP-1 and TIMP-3 overexpression were investigated. Elevated MMP inhibitory activity was demonstrated in the conditioned medium and cell lysates of AdTIMP-1- and AdTIMP-3-transduced rheumatoid synovial fibroblasts compared with AdLacZ-transduced fibroblasts. Moreover, overexpression of TIMP-3 by AdTIMP-3 gene transfer inhibited the activation of pro-MMP-2 in the conditioned medium, as shown by gelatin zymography. Pro-MMP-2 is mainly activated at the cell surface by MT-MMP.[28] Therefore, the inhibition of MMP-2 activation in the AdTIMP-3-transduced cells is most likely due to inhibition of MT-MMP. In an *in vitro* invasion model, the invasion of rheumatoid synovial fibroblasts through a Matrigel® or collagen I matrix could be significantly inhibited by both AdTIMP-1 and AdTIMP-3 gene transfer. The results in the *in vivo* cartilage SCID mouse co-implantation model also showed a clear reduction of invasion into the cartilage by the rheumatoid synovial fibroblasts that were transduced with AdTIMP-1 or AdTIMP-3.

The significant reduction of invasion of rheumatoid synovial fibroblasts by overexpression of TIMP-1 suggests that MMPs other than MT-MMPs are important mediators of invasion. However, the finding that overexpression of TIMP-3 resulted in a significantly stronger reduction of invasion stresses the importance of the properties of TIMP-3 that are distinct from those of TIMP-1. The ability to inhibit MT-MMPs and the association to the extracellular matrix[29] may explain the stronger effect of TIMP-3. The abundant presence of MT-MMPs at the invasive front of the pannus tissue in RA patients compared with the synovial tissues of patients with osteoarthritis or without arthritis[8,10,30] supports the view that MT-MMPs play an important role in cartilage destruction in RA. MT-MMPs are capable of degrading cartilage macromolecules including aggrecan[31] and collagen II.[32] Moreover, they activate other MMPs, such as MMP-2 and MMP-13.[28] Since active MMP-2 and MMP-13 are both implicated in the pathogenesis of joint destruction in RA,[8,9,13] the inhibition of the activation of these enzymes by TIMP-3 overexpression may be an efficient way to inhibit MMP-mediated cartilage invasion. The property to bind to the extracellular matrix may also contribute to the inhibitory effect of TIMP-3, since it keeps the inhibitor localized to the direct environment of the invasive cells.

To investigate effects of AdTIMP-1 and AdTIMP-3 gene transfer besides MMP inhibition that may influence the invasiveness of rheumatoid synovial fibroblasts, the effects on cell viability and cell proliferation were studied. Using less than 500 PFU/cell, no significant effects of AdITMP-1 or AdTIMP-3 gene transfer on cell viability was noted. Interestingly, cell proliferation was significantly inhibited by AdTIMP-3 gene transfer as compared with AdLacZ gene transfer and to a lesser extent by AdTIMP-1 gene transfer. The effects of TIMP-1 and TIMP-3 overexpression on proliferation differ per cell type. An inhibitory effect of TIMP-1 overexpression on cell proliferation was observed in hepatocytes[33] and of TIMP-3

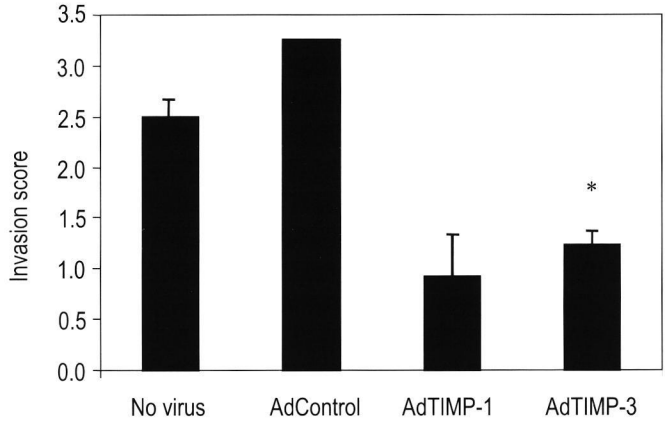

Figure 8. Cartilage invasion by rheumatoid synovial fibroblasts is inhibited by TIMP-1 and TIMP-3 gene transfer in the SCID co-implantation mouse model. *The depth of invasion of rheumatoid synovial fibroblasts that were co-implanted with human articular cartilage under the renal capsule of SCID mice was scored*

overexpression in colon carcinoma cells,[34] but in smooth muscle cells, no effect of TIMP-1 overexpression was observed.[20] In TIMP-3 overexpressing smooth muscle cells an accumulation of the cells in the S and G2/M phase of the cell cycle was reported in combination with an increase in cell death by apoptosis.[20] The mechanisms by which TIMPs effect the cell cycle are unclear. Since administration of exogenous MMP inhibitors has been shown to reduce tumor growth, it has been suggested that inhibition of proliferation may be caused by inhibition of degradation of extracellular matrix molecules which regulate the cell cycle by cell-matrix interactions.[20] However, this may not explain the inhibition of proliferation in the AdTIMP-1- and AdTIMP-3-transduced cells that was observed in the present study, since the cells were cultured in a monolayer on plastic. It has been demonstrated that the inhibitory effects of TIMP-2 overexpression on the proliferation of smooth muscle cells could not be mimicked by the addition of recombinant TIMP-2 to the cell culture,[20] suggesting that the cell cycle effects of overexpression of TIMPs may be caused by intracellular mechanisms. Culturing rheumatoid AdTIMP-1- and AdTIMP-3-transduced rheumatoid synovial fibroblasts revealed that transgenic TIMP-1 and TIMP-3 were still expressed after 60 days, suggesting that a large part of the rheumatoid synovial fibroblasts survived AdTIMP-1 and AdTIMP-3 transduction. Moreover, for TIMP-1 there was a stable overexpression as measured by ELISA throughout the 60 days culture period. This suggests that the reduced invasion of the AdTIMP-1- and AdTIMP-3-transduced cells observed in the SCID mouse co-implanation model after 60 days was due to stable overexpression of TIMP-1 and TIMP-3 resulting in effective inhibition of MMP-mediated invasion.

The effects of intravenous injection of adenoviral constructs encoding TIMP-1 has been tested in collagen-induced arthritis (CIA) in mice and in TNF-α overexpressing mice that

develop polyarthritis spontaneously. In TNF-α-overexpressing mice, a reduction of both joint destruction and inflammation by intravenous AdTIMP-1 gene transfer was demonstrated.[35] In contrast, in CIA no beneficial effect of TIMP-1 overexpression was noted.[36] In fact, TIMP-1 overexpression tended to worsen inflammatory scores and joint erosions. This is in contrast to results of another study investigating the effects of intravenous injection of recombinant TIMP-1 mice with CIA, showing a decreased incidence of joint erosions in the TIMP-1 treated mice.[37] These conflicting results of TIMP-1 overexpression in arthritis models suggest that the efficacy of TIMP-1 in arthritis may depend on factors such as the arthritis model that is used, the intra-articular TIMP-1 concentration that is achieved, and the time point on which the treatment is started. The augmentation of arthritis that was observed in mice with CIA[36] suggests that TIMP-1 may also have proinflammatory effects, which are unwanted for the treatment of an inflammatory disease such as RA. Overexpression of TIMP-3 may be a better choice. The results presented here show that the invasiveness of rheumatoid synovial fibroblasts is effectively inhibited by TIMP-3 overexpression. Moreover, other effects of TIMP-3, such as the reduction of proliferation and the inhibition of TACE, which processes membrane-associated TNF-α to a soluble form,[38] may have beneficial effects on synovial inflammation.

In conclusion, overexpression of TIMP-1 and TIMP-3 by adenoviral gene transfer results in a marked reduction of the invasiveness of rheumatoid synovial fibroblasts *in vitro* and *in vivo*. *In vitro*, TIMP-3 overexpression showed a stronger inhibitory effect than TIMP-1, which may be caused by distinct properties of TIMP-3 including the inhibition of MT-MMPs and the binding of TIMP-3 to extracellular matrix molecules. In addition to the inhibition of MMPs, the reduction of proliferation, demonstrated in AdTIMP-3- and, to a lesser extent, in AdTIMP-1-transduced cells, may also contribute to the inhibition of invasion. These results suggest that overexpression of TIMPs, particularly TIMP-3, at the invasive front of the pannus tissue may provide a novel therapeutic strategy to inhibit joint destruction in RA.

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7

LACK OF EFFECT OF DOXYCYCLINE ON DISEASE ACTIVITY AND JOINT DAMAGE IN PATIENTS WITH RHEUMATOID ARTHRITIS. A DOUBLE-BLIND PLACEBO-CONTROLLED TRIAL

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Journal of Rheumatology 2001;28:1967-1974

Summary

Matrix metalloproteinases (MMPs) are implicated in the pathogenesis of joint destruction in rheumatoid arthritis (RA). In the present study, the effects of doxycycline, an inhibitor of MMPs, on disease activity and joint destruction in patients with rheumatoid arthritis (RA).

A 36-week double-blind, placebo-controlled cross-over trial was conducted. Patients (n=66) received 50 mg doxycycline or a placebo twice a day during 12, 24, or 36 weeks. Patient assessments were performed before the treatment was administered, at 6, 12, 24, and 36 weeks of treatment, and finally at 4 weeks after cessation of the treatment. Patient assessments, swollen and tender joint counts, duration of morning stiffness, ESR and the Modified Disease Activity Score were used as parameters of disease activity. The effects on joint destruction were assessed by the urinary excretion of pyridinolines hydroxylysylpyridinoline and lysylpyridinoline and by scoring radiographic damage of hands and feet before and after treatment.

The observed changes of clinical or laboratory parameters of disease activity, pyridinoline excretion or progression of radiographic joint damage during doxycycline or placebo treatment did not differ significantly.

In conclusions, the results of the present double-blind placebo-controlled study indicate that 50 mg doxycycline twice a day provides no therapeutic benefit for RA patients.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease involving multiple joints, leading in most cases to irreversible destruction of the articular cartilage and bone. For RA patients, joint destruction is an important determinant of functional capacity.[1] Arresting the progression of joint destruction is therefore an important target in the treatment of RA.

Proteolytic enzymes secreted at the site of destruction cause degradation of the articular cartilage and bone. Although all classes of proteolytic enzymes seem to be involved, cartilage destruction has especially been attributed to matrix metalloproteinases (MMPs) and serine proteases.[2-4] The MMPs are a family of proteolytic enzymes that have the ability to degrade almost all proteins of the extracellular matrix. For example, collagenases (MMP-1, -8, -13) can degrade intact collagen fibrils, gelatinases (MMP-2, -9) can degrade unwound collagen, and stromelysins (MMP-3, -10) can degrade proteoglycans. In rheumatoid synovial tissue, increased amounts of MMPs are expressed.[5-7] MMP levels in the synovium and serum are correlated with disease activity[8] and radiographic damage,[9] suggesting a role for MMPs in the destructive process in RA. Therefore, inhibition of MMPs may be a therapeutic strategy to prevent joint destruction in RA.

As an unrelated function to their antibiotic activity, tetracyclines, in particular minocycline and doxycycline, are potent inhibitors of MMPs.[10-12] In patients with joint disease, a significant reduction of MMP activity within the joints was demonstrated after oral administration of minocycline[13] or doxycycline.[14] Double-blind placebo-controlled studies show that minocycline relieves clinical symptoms and reduces laboratory parameters of disease activity in patients with RA.[15-17] However, in these studies the progression of radiographic damage was not significantly reduced.[15,18] Moreover, treatment with minocycline may cause specific adverse effects. Dizziness is frequently reported[15,17] and led in a few cases even to fractures.[15] Rare but potentially serious adverse reactions to minocycline are drug-induced autoimmune syndromes such as a lupus-like syndrome[19,20] or autoimmune hepatitis.[20] In almost all cases the symptoms subside after discontinuation of minocycline, but cases of lethal minocycline-induced autoimmune hepatitis have been described.[20] The lack of effect of minocycline on the progression of joint damage combined with its risk of adverse effects led to an investigation of the therapeutic effects of doxycycline in patients with RA.

To date, only a few clinical studies have evaluated doxycycline as an anti-rheumatic medicine.[21,22] A small uncontrolled pilot study involving 13 patients treated with low dose doxycycline (20 mg twice a day) revealed that urinary pyridinoline excretion was reduced in patients with moderate, but not severe RA.[21] These results are in line with observations in rats with adjuvant arthritis showing that elevated pyridinoline excretion was reduced by doxycycline.[23] Pyridinolines are collagen cross-links that are released from the joints during cartilage and bone resorption and are finally excreted in the urine. Urinary pyridinoline excretion rates are increased in patients with RA[24-26] and correlate with the severity of joint

destruction.[27] The observed reduction in pyridinoline excretion rates suggest that doxycycline may have a joint protective effect in RA patients. In a recent study, a significant reduction in the number of tender joints and a significant improvement of domestic disability and vocational behaviour were reported in RA patients during doxycycline treatment.[22]

Because both studies were not placebo-controlled, it cannot be ruled out that the observed effects were due to other factors than the doxycycline treatment. Therefore, the present double-blind placebo-controlled study was designed. The aim of this study was to determine whether 12, 24 or 36 weeks of adjuvant therapy with doxycycline can ameliorate clinical symptoms in RA, can reduce the excessive excretion of pyridinolines, and whether it can slow down the progression of radiographic joint damage.

Patients and methods

Patients

Sixty-six patients from the outpatient clinics of the Leiden University Medical Center Leiden and The Jan Van Breemen Institute Amsterdam with active RA according to the 1987 criteria of the American Rheumatism Association[28] were included. Further inclusion criteria were: age between 18 and 85 years; stable second-line therapy for at least 10 months prior to the study; a daily dose of corticosteroids of less than 7.5 mg; no intra-articular steroid injections during the period of 1 month prior to the trial; and a baseline score of at least 3 on the prognostic index (PI) and of at least 5 on the disease activity index (DAI). The PI and DAI were used to include patients with high disease activity who were likely to have high baseline levels of urinary pyridinoline excretion rates, since the levels of pyridinolines are correlated to levels of disease activity.[25,27] To calculate the PI the sum of the presence of the following parameters were taken: radiographic joint damage, extra-articular manifestation (nodules, serosal disease, conjunctivitis or iridocyclititis), ESR > 30 for 6 months, CRP >6, and deteriorating function.[29] TheDAI was calculated by taking the sum of the scores (0=mild; 1=moderate; 2=severe) for the duration of morning stiffness, tender joint counts, swollen joint counts and ESR.[29] Patients with Steinbrocker functional stage of IV, impaired renal or hepatic function, active oesophago-gastro-duodenal ulcer, or women who were pregnant, lactating or planning to become pregnant were excluded from the trial.

The sample size was calculated for the expected differences in the changes of urinary HP and LP excretion rates. Based on previous studies,[21,23] we aimed at a decrease of 40% in the doxycycline treated group compared to 0% change in the placebo treated group. With a power of 80% and a significance of 5%, 16 patients per group were required. We included 20 patients per group, but in each group several patients dropped out before the study medication was started. Unfortunately these dropouts were not symmetrically distributed over the four groups and were left out of the analysis. This left us with 15 patients in group A, 17 patients in group B, 16 patients in group C, and 18 patients in group D.

Study design

The local institutional ethics committees approved the study protocol. The trial was set up as a 36-week, double-blind, placebo-controlled crossover trial. After informed consent, patients were treated in 3 consecutive treatment periods of 12 weeks with either doxycycline (50 mg twice a day) or a placebo. The patients were randomly divided over 4 treatment groups: A. doxycycline (week 1-12), placebo (week 13-24), doxycycline (week 25-36); B. doxycycline (week 1-24), placebo (week 25-36); C. doxycycline (week 1-36); D. placebo (week 1-36). The patients visited the outpatient clinics on 8 occasions: 2 weeks and 1 week before the start of the study, on the first day of the study period, at 6, 12, 24, and 36 weeks during the study period, and at 4 weeks after the study period. The cross-over design was used to account for the great variability in pyridinoline excretion. In this design, each patient could be his or her own control. Furthermore, this study design allowed us to compare the effects of doxycycline in various treatment periods (12, 24, and 36 weeks) and to document possible rebound effects after each treatment period.

No alterations in DMARD regimens and no intra-articular injections were allowed during the trial. Concomitant therapies are listed in table 1. Doxycycline and placebo were prepared in identical capsules and were added to the patients' therapy. The trial medication was one 50 mg capsule twice a day. Compliance was checked by pill counting.

Patients were interviewed about the appearance of new symptoms, gastrointestinal adverse effects, skin rash and photosensitivity. In addition the patients were asked to mention other adverse reactions they attributed to the study drug treatment.

Clinical assessments

At inclusion the following demographic and disease characteristics were noted: sex, age, disease duration (years), the presence of erosions, the presence of a positive IgM rheumatoid factor (RF; defined as >30 U/ml), and the use of NSAIDs, DMARDs or corticosteroids. At each visit, the following clinical assessments of disease activity were performed: a patient overall assessment of current disease activity on a visual analogue scale (VAS) of 0-100 mm, the duration of morning stiffness (minutes), swollen and tender joint count (28 joints), and the modified disease activity score (m-DAS) using 28 joint counts.[30]

Laboratory assessments

At each visit, serum and urine were collected and stored in –80 °C until use. The ESR (mm/h) was measured and urinary pyridinolines hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) were measured using HPLC.[31] Normal values for healthy people were assessed in a group of 36 adults. To assess hepato-renal toxicity, serum alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, γ-glutamyltranspeptidase and creatinin were measured at each visit.

Radiographic assessment

Radiographs of the hands and feet were made before study entry and repeated at the end of the study. All radiographs were scored separately in chronological order by the Sharp method, modified by van der Heijde[32] by the same rheumatologist who was uninformed of the treatment received by the patients.

Statistical analysis

Pearson Chi-square tests were used to test for differences between the treatment groups in sex, presence of erosions, RF, the usage of NSAIDs, DMARDs or corticosteroids, the number of patients who reported adverse effects, and the number of patients who discontinued the study. One-way analysis of variance was performed to test for differences in age and in baseline values of tender and swollen joint counts, VAS, mDAS, ESR, HP, and LP between the treatment groups. For HP and LP a logarithmic transformation was performed to obtain normally distributed values. Kruskall-Wallis tests were performed to test for differences in disease duration and Sharp score at baseline between the treatment groups. The efficacy parameters were analysed on the basis of an intention-to-treat analysis. For patients who prematurely discontinued the study, the data from the visit at which the trial medication was stopped were carried forward. Differences between courses of all outcome parameters except for the Sharp score during the treatment period were evaluated by repeated measure analysis of variance. The differences between the treatment groups in the changes of the Sharp score were tested by one-way analysis of variance. The changes (Δ) of all parameters during 12, 24 and 36 weeks of doxycycline treatment were compared with the changes in the placebo group. Differences were tested for significance by Student's t-tests. All statistic calculations were performed using SPSS 10.0 for Windows. P values of less than 0.05 were considered to be statistically significant.

Results

Sixty-six patients were included and randomly divided over 4 treatment groups. The groups were not statistically different at baseline with respect to demographic and disease characteristics (Table 1). In group C the baseline Sharp score was higher than in the other groups, but the difference was not significant (Kruskal Wallis; p=0.2). The patient groups did not differ in the usage of DMARDs. The usage of NSAIDs at baseline differed significantly between groups (Pearson chi square; p=0.008). In group C and D all patients used NSAIDs. In group A 10 (67%) patients and in group B 13 (77%) used NSAIDS. More patients in group A used corticosteroids (5 patients) at baseline as compared to the other treatment groups (0-1 patients; Pearson Chi-square; p=0.05; Table 1). There were 22 premature discontinuations: 5 in group A, 7 in group B, 2 in group C, and 8 in group D (placebo). In group A, one patient discontinued in week 5 during doxycycline treatment because of increase in disease activity;

one patient in week 12 at the end of doxycycline treatment for unknown reason; three patients in week 24 at the end of placebo treatment because of lack of effect. In group B, all dropouts were in the period they received doxycycline: one patient because of nausea at week 6, four because of lack of effect at week 12 and 24, one patient because of an intra-articular injection of corticosteroids at week 24, and one patient for unknown reasons at week 24. In group C, one patient discontinued at week 24 because of lack of effect and one patient discontinued because of gastrointestinal complaints at week 12. In group D, four patients discontinued due to lack of effect: one patient discontinued at 2 weeks, one at 12 weeks, and two patients at 24 weeks, one patient discontinued at 24 weeks because of intra-articular corticosteroid injection, one patient discontinued at week 12 because of gastrointestinal complaints, and one patient discontinued because of surgery.

	Treatment group			
	A (n=15)	B (n=17)	C (n=16)	D (n=18)
Males / females	8 / 7	5 / 12	7 / 9	3 / 15
Age: yrs, mean (sd)	62 (16)	56 (11)	53 (11)	57 (9)
Disease duration:				
yrs, median (range)	7 (1-25)	9 (3-22)	12 (4-47)	9 (1-47)
Erosive disease + / -	14 / 0	17 / 0	16 / 0	17 / 1
Sharp score: median (range)	91 (4-324)	50 (4-413)	180 (23-339)	52 (0-370)
RF + / -	15 / 0	15 / 2	13 / 3	14 / 4
PI , median (range)	3 (3-5)	3 (3-5)	3.5 (3-5)	4 (3-5)
DAI, median (range)	7 (5-8)	6 (5-8)	6 (5-8)	6 (3-8)
Drugs during study: n (%)				
NSAIDs	10 (67)*	13 (77)*	16 (100)	18 (100)
DMARDs	12 (80)	17 (100)	14 (88)	17 (94)
Antimalarials	3 (20)	2 (12)	2 (13)	3 (17)
Sulphasalazine	5 (33)	8 (47)	6 (38)	8 (44)
Methotrexate	2 (13)	2 (12)	1 (6)	2 (11)
Gold	2 (13)	3 (19)	4 (25)	0 (0)
Azathioprine	0 (0)	4 (24)	1 (6)	4 (25)
Corticosteroids	5 (30)*	0 (0)	1 (6)	1 (6)

Table 1. Characteristics of the patients at the start of the study in the different treatment groups *Treatment schedules: A. doxycycline-placebo-doxycycline; B. doxycycline-doxycycline-placebo; C. doxycycline-doxycycline-doxycycline; D. placebo-placebo-placebo.*
** Pearson Chi square; $p<0.05$.*

Changes in serum levels of the liver enzymes or creatinin during the study were no reason in any case to discontinue the study medication. Forty-four patients completed the entire study period.

Adverse effects were gastrointestinal and were reported in all treatment groups including the placebo group (Table 2). There was no significant difference in the number of patients reporting adverse effects between the treatment groups. In the doxycycline-treated groups the adverse effects occurred during treatment with doxycycline and not during treatment with the placebo.

	Treatment groups			
	A (n=15)	B (n=17)	C (n=16)	D (n=18)
Patients reporting adverse effects (n)	2	2	4	3
Reported symptoms (nr)				
Nausea	1	2	1	0
Stomach-ache	1	0	1	3
Diarrhoea	0	0	3	0
Total	2	2	5	3

Table 2. Adverse effects during the 36 week study period. *Treatment schedules: A. doxycycline-placebo-doxycycline; B. doxycycline-doxycycline-placebo; C. doxycline-doxycycline-doxycycline; D. placebo-placebo-placebo. All adverse effects in treatment groups A, B, and C were reported during doxycycline treatment. One patient in group C reported both stomach-ache and diarrhoea and discontinued the study after 12 weeks. In one patient in group B nausea led to discontinuation of the study drug treatment within 6 weeks of the study. One patient in group D (placebo) discontinued the study due to stomach-ache after 12 weeks.*

No effects of doxycycline on clinical parameters of disease activity or ESR

To evaluate the effect of doxycycline in RA, parameters of disease activity (patient global assessment, mDAS, and ESR) were determined at the start, at every 12 weeks and at 4 weeks after discontinuation of the study drug treatment. For all outcome parameters at baseline, there were no significant differences between the treatment groups.

Repeated measures analysis of variance showed no significant differences between the treatment groups in the courses of the patient global assessment, swollen joint count, tender joint count, mDAS, duration of morning stiffness, or ESR. Analysis of the changes (Δ) of these disease activity parameters at the start and the end of the 12, 24 or 36 week treatment period showed no or little difference between patients in the different treatment groups (Table 3; Figure 1).

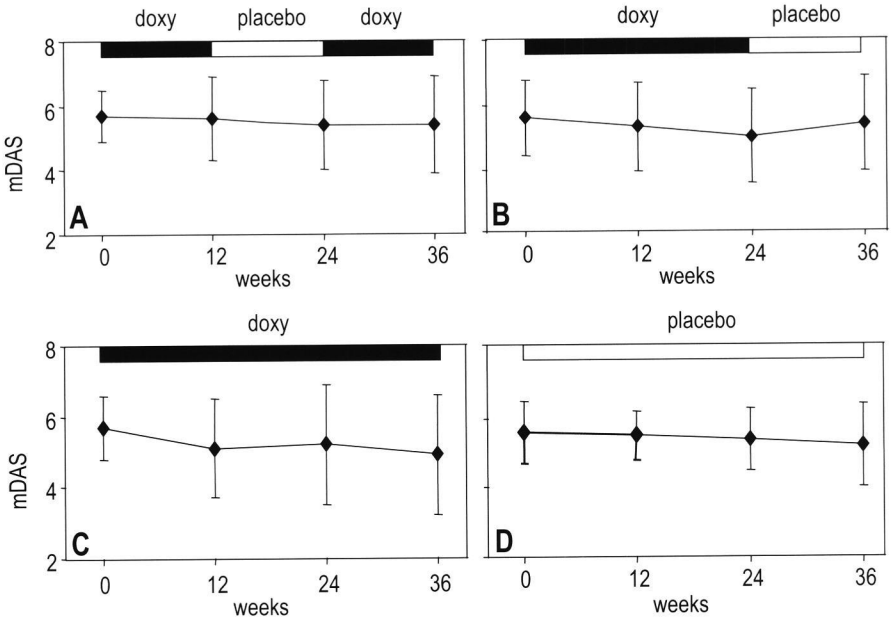

Figure 1. No effect of doxycycline on disease activity. *The effects of doxycycline in three treatment regimens on the courses of the modified DAS were evaluated and compared to the placebo. DAS scores are depicted as means and standard deviations.*

No effects of doxycycline on pyridinoline excretion rates or progression of radiographic damage

To assess the effects of doxycycline on the progression of joint destruction urinary HP and LP excretion rates were measured at the start, at every 12 weeks and at 4 weeks after discontinuation of the study drug treatment and radiographs of hands and feet were made before and after the study period. There were no significant differences in HP and LP excretion rates at baseline between the treatment groups. Normal values in healthy volunteers were <40 nmol/mmol creatinin for HP and <10 nmol/mmol creatinin for LP. The urinary excretion rates of HP and LP were above normal values for 90% of the patients participating in this study. Repeated measures analysis of variance showed no significant differences between the groups in the courses of the excretion rates of HP and LP during the study period. Analysis of the changes (Δ) of the urinary excretion rates of HP and LP at the start and the end of the 12, 24 or 36 week treatment period showed no differences between patients treated with doxycycline or placebo (Table 3; Figure 2). Subgroup analysis of patients with high pyridinoline excretion rates at baseline (HP excretion rates >80 nmol/mmol creatinin) did not reveal any effect of doxycycline on HP or LP excretion rates either.

There was no significant difference in the progression of the Sharp score between the treatment groups (figure 3).

Mean±sd			VAS (mm)	TJ (nr)	DAS (score)	ESR (mm/h)	lnHP (mmol/ mol cr)	lnLP (mmol/ mol cr)
0-12 weeks								
Doxy (n=48)								
baseline	49.9±21.5	64.9±61.9	12.8±5.8	9.9±7.0	5.7±1.0	38.3±26.6	4.4±0.6	2.6±0.6
Δ	-3.5±18.4*	-0.6±48.7	-0.6±5.4	-1.4±6.8	-0.3±1.1	-3.6±12.6	0.0±0.3	0.0±0.4
Placebo (n=18)								
baseline	42.1±20.7	55.6±64.4	12.2±5.9	9.3±6.9	5.6±0.9	34.7±18.7	4.2±0.3	2.6±0.5
Δ	8.3±17.4	-21.9±45.3	-0.1±4.4	-0.8±5.4	0.0±0.8	-1.6±16.6	-0.0±0.3	0.1±0.2
0-24 weeks								
Doxy (n=33)								
baseline	46.0±20.8	62.0±60.3	12.8±5.8	10.7±7.2	5.6±1.0	36.0±23.3	4.3±0.5	2.5±0.5
Δ	-5.8±19.1	-4.8±55.9	-1.45±5.9	-2.5±8.6	-0.5±1.4	-4.2±17.6	0.0±0.3	0.0±0.3
Placebo (n=18)								
baseline	42.1±20.7	55.6±64.4	12.2±5.9	9.3±6.9	5.6±0.9	34.7±18.7	4.2±0.23	2.6±0.5
Δ	3.8±17.8	-16.7±54.5	-0.1±3.8	-1.7±5.2	-0.1±0.8	1.8±19.2	0.0±0.5	0.1±0.3
0-36 weeks								
Doxy (n=16)								
baseline	48.1±9.7	65.0±60.8	13.7±7.1	11.8±7.9	5.6±0.9	34.5±24.5	4.4±0.5	2.6±0.5
Δ	0.8±20.8	-1.3±50.5	-3.3±7.6	-3.6±9.0	-0.7±1.4	-2.8±15.1	0.0±0.3	0.1±0.4
Placebo (n=18)								
baseline	42.1±20.7	55.6±64.4	12.2±5.9	9.3±6.9	5.6±0.9	34.7±18.7	4.2±0.3	2.6±0.5
Δ	3.4±22.5	-13.6±47.6	-0.1±3.1	-1.9±7.2	-0.2±1.0	-2.9±15.6	-0.0±0.6	0.1±0.3

Table 3. Effects of doxycycline on disease activity or joint destruction parameters. *The effects of 12, 24, and 36 weeks treatment periods of doxycycline on the change (Δ) of parameters of disease activity (ESR, swollen joint count [SJ], tender joint count [TJ], VAS, DAS, and duration of morning stiffness [MS]) and on parameters of joint destruction (lnHP, and lnLP) were evaluated and compared to placebo. Baseline and Δ levels are presented as mean±sd. * Student's T-test $p<0.02$.*

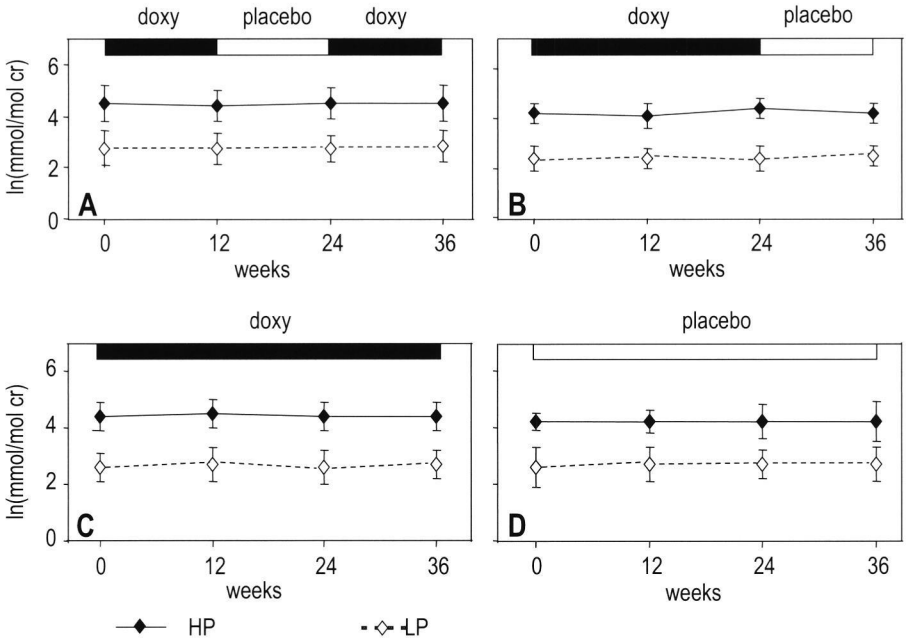

Figure 2. No effect of doxycycline on urinary excretion rates of pyridinolines. *The effects of doxycycline in three treatment regimens on the courses HP and LP were evaluated and compared to theplacebo. To obtain normally distributed parameters a logarithmic transformation was applied. HP and LP values are depicted as means and standard deviation of lnHP and lnLP.*

Discussion

Ample evidence on MMP inhibitory effects of doxycycline[10,12,14,14,22,33] and the generally accepted concept of the involvement of MMPs in joint destruction in RA[2,34] led to the hypothesis that doxycycline may be beneficial for RA patients as a joint protective medicine. In contrast to previous open-label studies describing an effect of doxycycline on urinary pyridinoline excretion rates[21] or on joint tenderness and disability parameters,[22] no significant effects of doxycycline on disease activity parameters or pyridinoline excretion rates were observed in the present study. Due to the size of the study small effects of doxycycline may have been missed. However, the results presented here do not indicate any clinically relevant effects of doxycycline that may have been observed in a larger study.

To achieve stable serum levels of doxycycline and a minimum of adverse effects, a dosage of doxycycline of 50 mg twice a day was chosen. Adverse effects, all gastrointestinal, were reported both during treatment with doxycycline and placebo. Stomachache was reported during both doxycycline and placebo treatment, suggesting that this adverse effect was not related to doxycycline treatment. Other adverse effects, such as nausea and diarrhoea, were reported during doxycycline treatment only, suggesting that these adverse effects may be related to doxycycline treatment. The number of patients reporting adverse effects or the number of discontinuations due to adverse effects did not differ significantly between the treatment groups. This suggests that in this patient group doxycycline in two daily doses of 50 mg does not produce significant adverse effects. In a previous study an effect of pyridinoline excretion rates in RA patients by as little as 20 mg doxycycline twice a day was reported.[21] In contrast, the results of the present study fail to confirm this effect of doxycycline in RA patients, even though a more than double dose of doxycycline was given. Moreover, a fifth of the patients treated with doxycycline discontinued the study prematurely due to lack of efficacy. This was similar to patients treated with the placebo.[35] This suggests that the effects observed in the open studies mentioned earlier[21,22] are likely to be due to other factors than to the doxycycline treatment.

The lack of effect on disease activity observed in the present study is in contrast to findings with minocycline. The effects of minocycline in RA have been extensively studied in double-blind placebo-controlled trials. Effects of minocycline (100 mg, twice a day) were observed on clinical symptoms and laboratory parameters reflecting disease activity such as improvement of swelling and tenderness of the joints,[17] patient and physician global assessment of disease activity,[16] CRP, haemoglobin, platelet count, and ESR.[15] None of these studies demonstrated a significant effect of minocycline on the progression of radiographic joint destruction. The discrepancy between effects of minocycline on disease activity and the lack of effect on joint destruction may be explained by other properties of tetracyclines besides MMP inhibition. Tetracyclines have various immunomodulating properties, such as the inhibition of the pro-inflammatory enzyme phospholipase A_2, suppression of neutrophils and T lymphocytes, anti-oxidative effects, inhibition of proliferation of peripheral blood lymphocytes,[36] inhibition of nitric oxide synthases,[37] and induction of apoptosis in activated T lymphocytes.[38] Even though doxycycline has such immunomodulating properties, no effects of doxycycline on disease activity measures were observed in the present study. An explanation may be that the dosage used in the present study was not sufficient to provide an anti-inflammatory effect as was demonstrated for minocycline. However, recent studies investigating the effects of doxycycline in intravenous doses of 200 and 300 mg over 12 weeks also fail to show effects of doxycycline on clinical and laboratory parameters of disease activity,[35,39] suggesting that even in higher doses doxycycline is not capable of suppressing the inflammation in RA patients. It is unclear why doxycycline, in contrast to minocycline, does not influence disease activity in rheumatoid arthritis. Perhaps differences

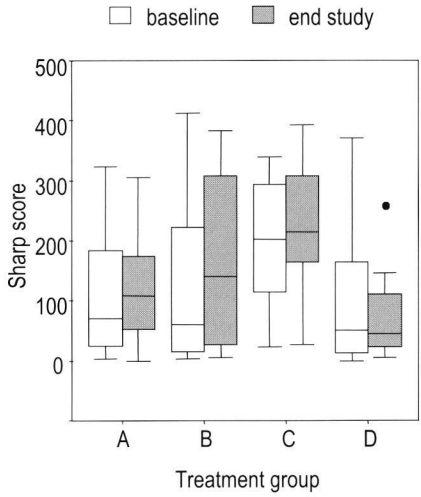

Figure 3. No effect of doxycycline on the progression of radiographic joint damage. *The effects of doxycycline in the different treatment groups on the progression of joint destruction was evaluated and compared to placebo. Radiographic damage was scored before study drug treatment (baseline) and at the end of the study period using the Sharp score, modification Van der Heijde. Sharp scores are depicted in Box-Whisker plots: horizontal line in box = median; limits of box = 25th and 75th percentiles; whiskers = highest and lowest values excluding outliers and extremes; • = outlier (between 1.5 and 3 box lengths from the upper or lower end of the box).*

in the chemical properties between minocycline and doxycycline, for instance minocycline being more lipophilic than doxycycline,[40] may result in a more favourable distribution of minocycline within the synovium. Moreover, in contrast to doxycycline, minocycline penetrates the blood-brain-barrier easily, which accounts for the frequently reported minocycline-induced vertigo.[41] One can speculate that, if minocycline interacts with parts of the central nervous system that influence the immune system,[42] this may contribute to its immunosuppressing effects.

The lack of effect of doxycycline and minocycline on joint destruction are disappointing, since results of studies describing potent inhibitory effects of tetracyclines on MMPs and on joint destruction both *in vitro* and *in vivo* appeared to be so promising.[43] An explanation for the lack of effect of minocycline and doxycycline on joint destruction may be that the dose regimens used in these studies did not provide sufficient MMP inhibitory levels within the joints to slow down the rate of joint destruction in RA patients. However, for minocycline it was demonstrated that 100 mg twice a day was sufficient to provide significant inhibition of collagenase activity in the synovial tissue cultures of RA patients.[13] For doxycycline, it was demonstrated that doxycycline in a dose of 20 mg twice a day achieved *in vivo* inhibition of excess MMP activity in patients with periodontitis.[44,45] Hence an effect of 50 mg doxycycline twice a day on MMP activity in the joints of RA patients was expected. Unfortunately, synovial fluid or biopsies of the patients in the present study were not obtained, so it cannot be ruled out that MMP inhibition in the joint by doxycycline was insufficient. Recent observations demonstrate that daily intravenous administration of as much as 200 or 300 mg doxycycline also produces no effects on cartilage and bone resorption,[35,39] suggesting that doxycycline even in high doses is not capable of inhibiting joint destruction in RA. Possibly, proteinases that are not inhibited by doxycycline are important mediators of joint destruction

in RA. MMP-1 and MMP-3 are not effectively inhibited by doxycycline,[14,46,47] whereas MMP-2, MMP-9, MMP-8, and MMP-13 are effectively inhibited in therapeutically attainable concentrations.[12,48-50] The inability of doxycycline to inhibit MMP-1 and MMP-3, both implicated in RA,[8,9] may explain why doxycycline was not effective as a joint protective drug in this study.

In conclusion, the results of the present study demonstrate that 12, 24, or 36 weeks of doxycycline in a dose of 50 mg twice a day does not relieve clinical symptoms, has no effect on the ESR and does not slow down the progression of joint destruction in RA patients. To achieve effective inhibition of joint destruction in RA, further studies need to be carried out investigating in what way the excess of proteolytic activity at the site of joint destruction is best inhibited.

Acknowledgement

The authors wish to thank Mrs. L.G.M. Huisman and Mr. A.N. Sakkee for their technical assistance, Professor A. Cats for scoring the radiographs, and Dr A.H. Zwinderman for help with the statistical analysis.

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8

SUMMARY AND CONCLUSION

In this chapter a summary of this thesis is given. Furthermore, the main findings and the perspectives for future studies are discussed.

Summary

Chapter 1 is a general introduction reviewing the current knowledge about the disease rheumatoid arthritis (RA), particularly with respect to the pathogenesis of joint destruction.

RA is a chronic inflammatory disease, which affects the synovial joints. The progressive, irreversible destruction of the joints is a serious feature RA, which may lead to severe disability of the patients. For the rheumatologist it is still a challenge to prevent or stop this process.
It has been suggested that the rheumatoid synovial fibroblast is an important player in the pathogenesis of joint destruction in RA. Rheumatoid synovial fibroblasts have "transformed cell-like" characteristics such as the expression of oncogenes, the loss of contact inhibition, and the ability to invade articular cartilage spontaneously. In contrast, osteoarthritis (OA) synovial fribroblast or dermal fibroblasts are not able to invade articular cartilage spontaneously. Moreover, the rheumatoid synovial fibroblasts can secrete growth factors and proinflammatory cytokines, which enhance the synovial inflammation.

Degradation of tissues such as cartilage, bone and tendons is caused by proteinases. Under normal conditions, proteinase activity is well regulated on the level of synthesis, activation of pro-enzymes, and by an abundance of inhibitors that are present within the tissues and the extracellular fluids. Under pathological conditions, for example rheumatoid synovium or a malignant tumor, the regulation is disturbed, which results in an excess of proteinase activity leading to degradation and invasion of the extracellular matrix. The plasminogen activator (PA) system and matrix metalloproteinases (MMPs) are believed to be involved in the pathogenesis of joint destruction in RA. Increased expression of components of the PA-system and MMPs within the rheumatoid synovium and the ability of these enzymes to degrade proteins of the articular cartilage and bone support this concept. Besides the PA-system and MMPs, it has been suggested that other proteinases, such as granzymes and cathepsins may also contribute to the pathogenesis of joint destruction in RA.

The goal of this thesis was contribute to the knowledge of the pathogenesis of joint destruction in RA in order to be able to design therapeutic strategies to prevent the development of damage to the articular tissues. Therefore, studies were conducted to 1) investigate the influence of chondrocytes on the invasiveness of the rheumatoid synovial fibroblast, 2) investigate the roles of the PA-system, MMPs, and granzymes in cartilage destruction, and 3) to explore therapeutic strategies to inhibit proteinase-mediated joint destruction.

Stimulation with macrophage-derived cytokines, particularly IL-1-β and TNF-α, contribute to the cartilage degradation and invasion by rheumatoid synovial fibroblasts. On the basis of the finding that chondrocytes can produce proinflammatory cytokines and factors such as IL-1-β, it has been hypothesized that chondrocytes may contribute to the pathogenesis of RA. However, direct

evidence of a significant role of chondrocytes in the cellular interplay in RA has thus far been lacking. The results in **Chapter 2** indicate that chondrocytes influence the invasive behavior of rheumatoid synovial fibroblasts. In the SCID mouse co-implantation model it was observed that the rate of invasion into articular cartilage by rheumatoid synovial fibroblasts decreased when cartilage was used that was stored for 1-2 days. *In vitro*, it was demonstrated that cartilage invasion by rheumatoid synovial fibroblasts decreased when protein synthesis in chondrocytes is inhibited. Adition of IL-1-β partially restored the invasiveness of rheumatoid synovial fibroblasts. This finding is in line with several studies demonstrating the potential of IL-1-β to stimulate cartilage degradation by synovial fibroblasts. Interestingly, the addition of TNF-α did not enhance the degradation of the cartilage-like matrix, suggesting that TNF-α is not involved in the interplay between chondrocytes and rheumatoid synovial fibroblasts.

In conclusion, the results in Chapter 2 demonstrate that chondrocytes influence the invasiveness of rheumatoid synovial fibroblasts by secreting factors including IL-1-β.

In **Chapter 3** the role of granzyme B in cartilage destruction in RA was investigated. Granzyme B is a serine proteinase which is secreted by natural killer cells and cytotoxic T-cells and which mediates apoptosis in target cells. In RA synovial tissue, a significantly higher number of granzyme B-positive cells was found than in synovial tissue of patients with reactive arthritis. When granzyme-containing granules are released from the cells, they may exert extracellular activity, such as proteolysis of extracellular matrix components. Previous studies show that granzyme B is able to degrade proteoglycans and that granzyme A is able to degrade collagen IV. In Chapter 3 it is demonstrated that granzyme B is capable of degrading proteoglycans directly from a newly synthesized cartilage matrix, as well as from intact articular cartilage, but incapable of degrading collagen. Immunohistochemistry of rheumatoid synovial tissues revealed that granzyme B-positive cells are present throughout the synovial tissue and at the invasive front of the pannus tissue. The granzyme B-containing granules were found both inside and outside the cells.

In a very recent study, a strong relationship was found between serum granzyme B levels and the development of erosions after a year in patients with recent-onset RA.[1] This suggests that the extracellular effects of granzyme B, such as the degradation of proteoglycans and other not yet elucidated effects may be important in the pathogenesis of joint destruction in RA.

The results described in Chapter 3 confirm that human granzyme B is capable of degrading the proteoglycan component of cartilage. This finding, together with the presence of granzyme B at the invasive front suggest that granzyme B may be involved in the pathogenesis of joint destruction in RA. Future studies may provide more insight into the way in which granzyme B may contribute to rheumatoid joint destruction, for instance by investigating the effects of specific inhibitors.

In **Chapter 4** the role of the plasminogen activator (PA) system in cartilage destruction in RA and the effects of gene transfer of plasmin inhibitors were investigated. The results indicate that

plasmin mediates the degradation of cartilage matrix by rheumatoid synovial fibroblasts and that its activation is mediated by urokinase-type plasminogen activator (uPA) that is secreted by the synovial fibroblasts themselves. In an *in vitro* model, it was possible to completely inhibit the degradation of a newly synthesized cartilage matrix by rheumatoid synovial fibroblasts, by using high concentrations of plasmin inhibitor BPTI. Partial inhibition was achieved by anti-uPA. In contrast, the addition of anti-tPA did not show an effect. Gene transfer with a cell surface-binding plasmin inhibitor, consisting of a plasmin inhibitor (BPTI) linked to the receptor-binding aminoterminal fragment of uPA (ATF), resulted in strong inhibition of cartilage matrix degradation *in vitro*. The property to bind to the uPA receptor (uPAR) at the cell surface contributed significantly to the inhibitory effect, as the effect of AdATF.BPTI gene transfer was significantly stronger than that of gene transfer of the non-binding inhibitor, AdBPTI, alone. Preventing uPA from binding to uPAR appeared not to contribute to the inhibitory effect of ATF.BPTI, since AdATF gene transfer did not affect synovial fibroblast-dependent cartilage matrix degradation at all, despite ATF levels at least as high as the ATF.BPTI levels measured after gene transfer. Targeting plasmin inhibition to the cell surface, however, did appear to be important as illustrated by a decrease in inhibitory activity when ATF.BPTI was prevented from binding to uPAR. This suggests that cell surface-bound plasmin may be important in cartilage degradation by rheumatoid synovial fibroblasts.

The effects of AdATF.BPTI gene transfer on cartilage invasion by rheumatoid synovial fibroblasts, were studied in the SCID mouse co-implantation model. In comparison with synovial fibroblasts transduced by the control vector, a significant reduction of the invasive growth into the cartilage was observed. Both the control and the AdATF.BPTI-transduced fibroblasts showed migration towards the edge of the cartilage, suggesting that the inhibitory effect of AdATF.BPTI gene transfer on invasion is not caused by impaired migration of the cells, but was most likely caused by impaired proteolytic degradation of the cartilage.

Altogether, the experiments described in Chapter 4 support the concept of the importance of the PA-system in joint destruction in RA and show that plasmin-mediated cartilage degradation and invasion by rheumatoid synovial fibroblasts can be effectively inhibited by gene transfer of the cell surface-targeted plasmin inhibitor, ATF.BPTI.

If joint destruction in RA is mediated by plasmin, inhibition of plasmin may be beneficial for RA patients. It has been shown that treatment with a plasmin antagonist, tranexamic acid, may have a joint protective effect, since a reduction of the urinary excretion rates of molecular markers of cartilage and bone degradation (i.e. collagen cross-links) was observed during tranexamic acid treatment in experimental arthritis and in an open study with RA patients.

To rule out the possibility that this effect was caused by other factors than tranexamic acid treatment, a double-blind placebo-controlled study was set up. The results of this study are described in **Chapter 5**. In contrast to the findings of the open study described above, no effects of tranexamic acid on urinary pyridinoline excretion rates were observed in this study, nor were any effects of tranexamic acid observed on parameters of disease activity.

It can be concluded that systemic inhibition of plasmin is not an effective therapeutic strategy to inhibit joint destruction in RA. Even though previous studies indicate that plasmin is directly involved in cartilage and bone destruction in RA, other proteinases, including MMPs and cathepsins, are also implicated. Therefore, inhibiting plasmin only may not be sufficient to inhibit joint destruction in RA patients. In that case, the observed decrease in pyridinoline excretion rates in the open study was caused by factors other than the treatment with tranexamic acid.

Another explanation may be that treatment with plasmin inhibitors is only effective in RA patients with very active disease. In the open study only patients with erosive disease with extensive synovial inflammation were included, whereas the disease activity in the patients who participated in the present study was mild or moderate. Moreover, in some of these patients erosions were absent. Since proinflammatory mediators, such as IL-1 and granulocyte macrophage colony stimulating factor increase the expression of uPA, it is conceivable that plasminogen activation is decreased in the synovial tissues of patients with low disease activity compared with those of patients with active disease.

Based on the findings of this study, it can be concluded that treatment with tranexamic acid is not beneficial as adjuvant therapy with respect to the inhibition of joint destruction in RA patients with mild or moderate disease activity.

In **Chapter 6** the role of MMPs in cartilage destruction in RA and the effects of gene transfer of tissue inhibitors of metalloproteinases (TIMPs) were investigated. The increased expression of MMPs in the synovial tissue of RA patients combined with the fact that MMPs are capable of degrading almost all proteins of the extracellular matrix, including articular cartilage, has led to the generally accepted view that an imbalance between MMPs and their physiologic inhibitors may be important in the pathogenesis of joint destruction in RA. The results described in Chapter 6 indicate that restoring this imbalance by overexpression of TIMPs results in a cartilage protective effect. Both in an *in vitro* invasion model and in the SCID mouse co-implantation model a clear reduction of cartilage invasion by AdTIMP-1 and AdTIMP-3 gene transfer was observed. The finding that TIMP-1, which is unable to inhibit MT-MMPs, significantly inhibits invasion indicates that MMPs other than MT-MMPs are involved in cartilage degradation. *In vitro*, a significantly stronger effect of gene transfer with AdTIMP-3 was observed than with AdTIMP-1, suggesting that properties of TIMP-3 that are distinct from TIMP-1, such as the inhibition of MT-MMPs and the binding to the extracellular matrix, contribute to the inhibitory effect. Besides MMP inhibition, TIMPs may also have other properties.

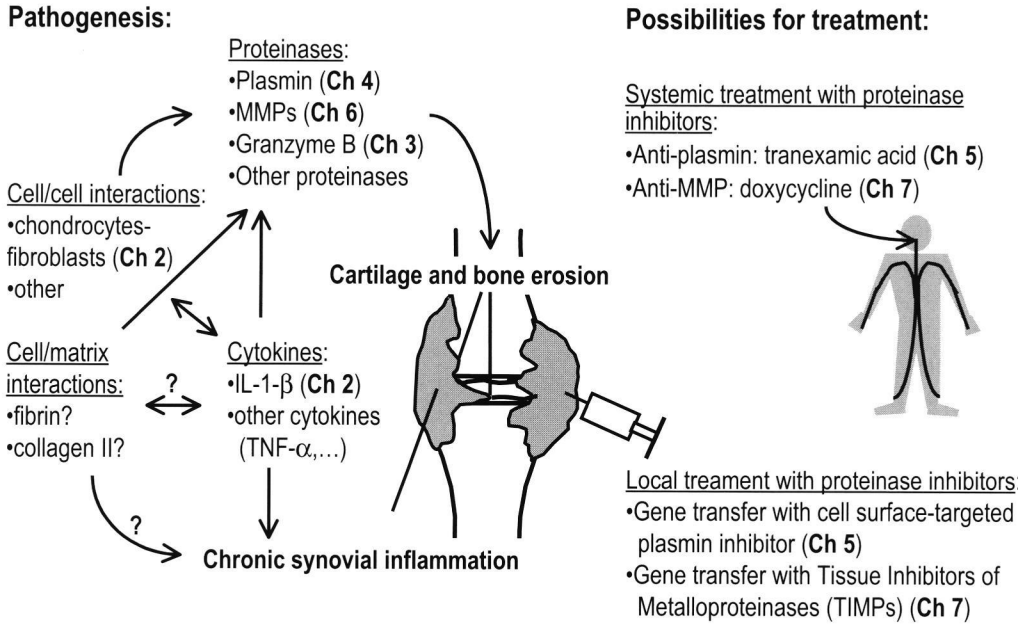
Here, an inhibitory effect on the proliferation of rheumatoid synovial fibroblasts by gene transfer with AdTIMP-3, and to a lesser extent with AdTIMP-1, was observed. Inhibition of proliferation combined with the property of TIMP-3 to inhibit the shedding of cell surface-bound TNF-α, resulting in less available soluble TNF-α, suggests that overexpression of TIMP-3 in the rheumatoid synovium may also be beneficial with respect to synovitis. Limiting the synovial hyperplasia by the inhibition of proliferation and reducing the abundant presence of TNF-α in the rheumatoid synovium may reduce the synovial inflammation.

The results described in Chapter 6 indicate that invasion of rheumatoid synovial fibroblasts into articular cartilage is mediated by MMPs and can be inhibited by gene transfer of AdTIMP-1 and AdTIMP-3. Apart from the inhibition of MMPs, a reduction in the proliferation rate may have contributed to this effect. These results suggest that overexpression of TIMPs, particularly TIMP-3, at the invasive front of the pannus tissue may provide a novel therapeutic strategy to inhibit joint destruction in RA.

Since MMPs appear to be important mediators of joint destruction in RA, inhibition of MMPs may be a therapeutic strategy to prevent or at least limit the progression of joint destruction in RA patients. Doxycycline belongs to the family of tetracyclines and is a common antibiotic. IN addition to an in a way unrelated to their antibiotic action, tetracyclines have been shown to be effective inhibitors of MMPs. The results of a two open studies showing a reduction of pyridinoline excretion rates in urine, as a measure of joint destruction, and effects on measures of disease activity in RA patients during treatment with doxycycline, led to investigations of the effects of doxycycline in a double-blind placebo-controlled study.

The results, as described in **Chapter 7,** demonstrate that doxycycline (50 mg twice a day) has no effect on measures of disease activity nor on parameters of joint destruction. An explanation of the lack of effect may be that the concentration of doxycycline within the synovial tissue was not sufficient to inhibit the excess of MMP activity at the invasive front of the pannus tissue. However, recent findings show that doxycycline administered in a very high concentration (daily 300 mg) intravenously is not able to protect the joints from destruction either. This suggests that doxycycline is not a suitable drug for inhibiting MMP-mediated joint destruction in RA. An explanation for the lack of effect of doxycycline may be that it does not inhibit the pivotal proteinases involved in the pathogenesis of joint destruction in RA. Possibly, proteinases that are not inhibited by doxycycline are important mediators of joint destruction in RA. For instance, MMP-1 and MMP-3, both implicated in RA, are less effectively inhibited by doxycycline than MMP-2, MMP-9, MMP-8, and MMP-13. Moreover, other proteinases that may be involved in the pathogenesis of joint destruction, such as plasmin and cathepins, are also not inhibited by doxycycline.

The results of the present double-blind placebo-controlled study indicate that 50 mg doxycycline twice a day provides no therapeutic benefit for RA patients. To achieve effective inhibition of joint destruction in RA, further studies need to be carried out investigating in what way the excess of proteolytic activity at the site of joint destruction is best inhibited.



Conclusion and perspectives

The pathogenesis of joint destruction in RA is complex and may be approached from different angles. In this thesis, we focused on the proteinases that are believed to be important in causing the damage to the articular tissues and investigated novel therapeutic strategies to inhibit these proteinases.

The results of the studies described in this thesis on the role of the PA-system and MMPs in cartilage degradation and invasion suggest that these enzyme systems are involved in the pathogenesis of joint destruction in RA. In addition, the results of the study on granzyme B support the idea that granzymes may also contribute to this process. To prevent joint destruction, treatment with inhibitors of these enzymes may be beneficial for RA patients. The goals of such treatment are to inhibit the excess of proteolytic activity at the site of joint erosion and to avoid systemic proteinase inhibition that may cause unwanted side effects. An unwanted effect of plasmin inhibition may be the inhibition of the dissolution of intra-articular fibrin depositions (see also below). Reported unwanted effects of MMP inhibitors include arthralgias, polyarthritis, and stiffening of the joints. The results of the clinical studies in this thesis investigating the effect

of the plasmin inhibitor tranexamic acid and of the MMP inhibitor doxycycline indicate that systemic proteinase inhibition may not be sufficient to inhibit proteinase-mediated joint destruction in RA. Therefore, localized treatment with proteinase inhibitors, may be a better approach. One can think of several possible ways to target proteinase inhibitors to the site of erosion. One possibility is by administering therapeutic genes directly into the affected tissue. Transcription of these therapeutic genes by the rheumatoid synovial cells results in local overexpression of certain inhibitors. The effects of gene transfer of plasmin - and MMP inhibitors in rheumatoid synovial fibroblasts were investigated in this thesis in *in vitro* as well as in *in vivo* models of rheumatoid cartilage destruction. So far, the results are promising: adenoviral gene transfer allows sufficient overexpression of active inhibitors to cause significant inhibition of cartilage destruction in these models. The results also show that by targeting plasmin inhibition to the cell surface of the destructive cells the inhibitory effect can be improved. In future studies the effect of targeting MMP inhibition to the cell surface may be investigated in order to improve the effects of MMP inhibitors, such as TIMPs. Another way of targeting proteinase inhibition to the inflamed synovium is for example by engineering T-cells that recognize collagen type II in the articular cartilage and carry the genes encoding proteinase inhibitors. Chernajovsky *et al.* are currently exploring such an approach.[2]

In this thesis it has been demonstrated that several enzyme systems, including the PA-system and MMPs, are likely to be involved in the pathogenesis of rheumatoid joint destruction. In addition, other proteinases, such as granzyme B, may also be involved. Therefore, it is not likely that inhibition of a single enzyme will effectively inhibit the destructive process in the rheumatoid joints. Further investigations need to be done to identify the key proteinases in rheumatoid cartilage and bone destruction, for example proteinases that activate other proteinases or stimulate proteinase expression, in order to design treatments with (a combination of) specific inhibitors. Candidate key enzymes are MT1-MMP, MMP-3, and plasmin since they are able to activate certain MMPs. Granzymes may also have a regulatory role. Little is known about the extracellular actions of granzymes, but for granzyme A it has been demonstrated that it is capable of inducing the expression of proinflammatory cytokines in synovial cells.

Another approach to explore possible ways of inhibiting rheumatoid joint destruction is to investigate the factors that cause the upregulation of tissue-degrading proteinases. In this thesis, we showed that chondrocytes influence cartilage invasion by rheumatoid synovial fibroblasts by secreting IL-1-β, which may result in an upregulation of proteinases such as uPA and MMPs. Other factors, including other cell-cell interactions, other proinflammatory cytokines, and cell-matrix interactions may also regulate the expression of proteinases within the rheumatoid synovium.

For example, the presence of fibrin depositions in the joint appears to enhance synovial inflammation and stimulate joint destruction. This is observed in animal models of arthritis showing that deficiency of the plasminogen activators tPA and uPA, results in more intra-articular fibrin, more severe synovitis and more severe joint destruction.[3,4] Elevated levels of

procoagulant factors within the joints suggest[5,6] that the presence of fibrin depositions is a result of increased fibrin formation. In addition, the dissolution of fibrin within the rheumatoid joint appears to be hampered due to inactivation of uPA by thrombin.[7] One can imagine that the presence of fibrin within the joints leads to an upregulation of plasminogen activators that on the one hand mediate the dissolution of fibrin (insufficiently) and on the other hand mediate the destruction of articular tissues. Simultaneously, the enhancement of intra-articular inflammation by fibrin may lead to upregulation of tissue-degrading proteinases through inflammatory mediators. One can think of therapeutic strategies to decrease the presence of intra-articular fibrin. Preventing fibrin formation may be one approach. This was investigated by Varisco *et al.* who found that treatment with the thrombin inhibitor hirudine had a somewhat beneficial effect on synovial inflammation and joint destruction in mice with antigen-induced arthritis.[8] In patients with established RA, who already have intra-articular fibrin depositions, one can think of a therapeutic strategy that enhances the dissolution of fibrin. The dual role of plasmin in the rheumatoid joint complicates the treatment with plasmin agonist or antagonists. Inhibition of plasmin-mediated joint destruction should not hamper the dissolution of fibrin deposition. Likewise, stimulation of fibrinolysis by plasmin should not enhance plasmin-mediated joint destruction. Targeting plasmin inhibition to the cell surface of synovial cells, for instance, by ATF.BPTI may be a way to reduce the formation of erosions without interfering in intra-articular fibrinolysis. When therapeutic strategies are designed in order to decrease intra-articular fibrin, possible effects on plasmin-mediated tissue degradation by synovial cells should be taken into account.

To summarize, the results of this thesis show that: 1) articular cartilage destruction by rheumatoid synovial fibroblasts is mediated by proteinases including the PA-system, MMPs, and granzyme B; 2) chondrocytes influence the invasiveness of rheumatoid synovial fibroblasts by the secretion of IL-1-β; 3) localizing inhibitors of these proteinases at the site of destruction, for instance by transfer of genes in the synovial fibroblasts encoding proteinase inhibitors, appears to be an effective way to inhibit proteinase-mediated cartilage destruction; 4) further improvement of the effectiveness of proteinase inhibitors can be achieved by targeting the inhibitor to the surface of the destructive cells.

These results, together with the work by others and further investigation, may lead to novel therapeutic strategies for RA patients that prevent the irreversible destruction of the joints.

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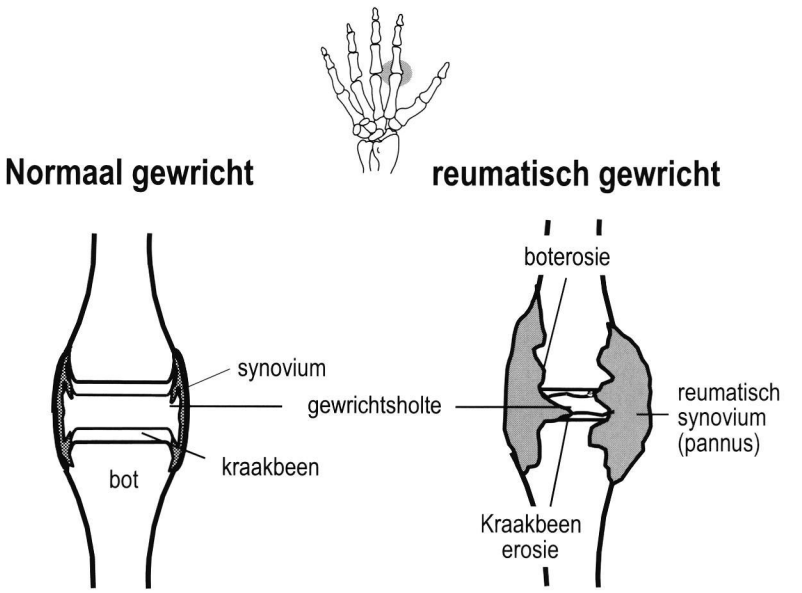
NEDERLANDSE SAMENVATTING

Reumatoïde artritis

Reumatoïde artritis is een ernstige, chronische ziekte, waarbij met name de gewrichten aangedaan zijn. Reumatoïde artritis behoort tot de reumatische aandoeningen (reuma), een verzamelnaam van ziekten van spieren of gewrichten, gekenmerkt door chronische, vaak verspringende pijn en ontstekingsverschijnselen. Circa 1% van de bevolking leidt aan reumatoïde artritis. Vrouwen zijn vaker aangedaan dan mannen. Reumatoïde artritis, in dit hoofdstuk kortweg aangeduid met reuma, kenmerkt zich door een chronische, periodiek opvlammende ontsteking van de binnenbekleding van het gewrichtskapsel, het synovium. Dit leidt tot pijnlijke, stijve gewrichten. Bij de meeste patiënten gaat de gewrichtsontsteking gepaard met toenemende, onherstelbare beschadiging van het bot, kraakbeen en pezen in en rondom de gewrichten. Hierdoor verliezen de gewrichten hun normale vorm (ze gaan scheef staan en/of vergroeien) en kunnen niet meer goed functioneren (Figuur 1). De pijn, de gewrichtsbeschadiging en de verschijnselen die met een chronische ziekte gepaard gaan, bijvoorbeeld vermoeidheid, maken dat reuma een invaliderende ziekte is met in veel gevallen (gedeeltelijke) arbeidsongeschiktheid tot gevolg.



Het immuunsysteem speelt een belangrijke rol bij het ontstaan van reuma. Wat precies het immuunsysteem aanzet om juist in het synovium een chronische ontstekingsreactie te veroorzaken is nog niet helemaal duidelijk. Erfelijke factoren spelen een rol, maar verklaren slechts ten dele waarom de ene persoon wel en de ander geen reuma krijgt.

De huidige behandeling van reuma is met name gericht op het remmen van de ontsteking. Met de nieuwste therapieën is het mogelijk bij de meeste patiënten de ontsteking redelijk tot goed onder controle te krijgen. Toch blijft het een uitdaging om het optreden van gewrichtsbeschadiging te voorkomen of verdere toename substantieel te remmen.

Gewrichtsdestructie bij reuma

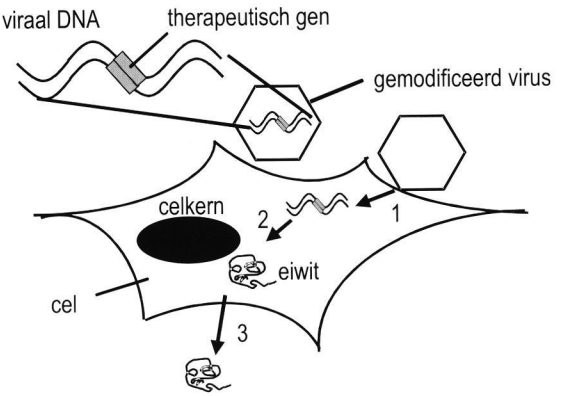
Bij de afbraak van weefsels, zoals kraakbeen en bot, spelen proteolytische (eiwitafbrekende) enzymen een belangrijke rol. Het lichaam heeft proteolytische enzymen nodig bij processen waarbij weefsels afgebroken en weer opgebouwd worden, zoals groei en wondgenezing. Om hun functie goed en op de juiste plaats uit te kunnen oefenen, worden proteolytische enzymen goed onder controle gehouden. Meestal worden ze in een inactieve vorm uitgescheiden en moeten de enzymen eerst geactiveerd worden voordat ze functioneel zijn. Bovendien is er een overmaat aan enzymremmers in het lichaam aanwezig, die de activiteit van proteolytische enzymen neutraliseren. Bij bepaalde ziektes, zoals kanker en reuma, is die regulatie verstoord. In een kankergezwel en in een door reuma aangedaan synovium wordt een grote hoeveelheid aan proteolytische enzymen geproduceerd en zijn er niet genoeg remmers om hun activiteit te blokkeren. Bij kanker kunnen hierdoor uitzaaiingen ontstaan. Bij reuma veroorzaakt de overmaat aan proteolytische enzymen afbraak van de weefsels in een aangedaan gewricht en invasie in het kraakbeen en het onderliggende bot door het aangedane synovium (Figuur 1).

Van een aantal enzymen is bekend dat ze bij dit soort processen een rol spelen en mogelijk ook bij reuma de gewrichtsdestructie veroorzaken. Plasmine en matrix metalloproteinases (MMPs) zijn enzymen, waarvan bekend is dat ze bij het uitzaaien van kwaadaardige tumoren een belangrijke rol spelen en in het synovium van reumapatiënten in verhoogde mate voorkomen. Het gegeven dat deze enzymen in staat zijn om bestanddelen van kraakbeen en/of bot af te breken wijst erop dat deze enzymen betrokken zouden kunnen zijn bij de destructie van de gewrichten van reumapatiënten. Er zijn ook aanwijzingen dat andere proteolytische enzymen, zoals granzymen en cathepsines een rol spelen bij het ontstaan van gewrichtsdestructie bij reuma.

Doel van dit proefschrift

Het doel van dit proefschrift was om 1) meer inzicht te krijgen in het mechanisme dat leidt tot onherstelbare schade aan de gewrichten ten gevolge van reuma; 2) te onderzoeken wat de rol van proteolytische enzymen zoals plasmine, MMPs en granzym B is in dit proces en 3) te onderzoeken op welke wijze de destructie geremd kan worden.

De onderzoeken die in het kader van dit proefschrift zijn uitgevoerd staan beschreven in de hoofdstukken 2 t/m 7. Hieronder worden twee aspecten die in de verschillende hoofdstukken terugkomen eruit gelicht en wordt een summiere samenvatting van de resultaten gegeven.



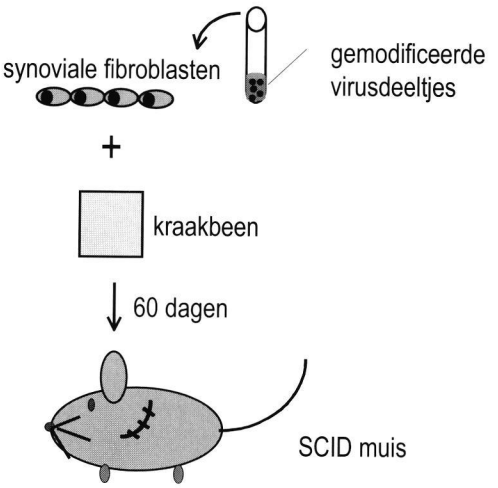
Figuur 2. Gentherapie. *1. Een genetisch aangepast virusdeeltje infecteert een cel en brengt zo het therapeutische in de cel. 2. Het gen wordt afgeschreven, zodat het therapeutische eiwit wordt gevormd. 3. Dit eiwit wordt vervolgens uitgescheiden en kan buiten de cel zijn functie vervullen, bijvoorbeeld het remmen van schadelijke enzymen.*

enzymremmers, is het belangrijk dat deze remmers op de juiste plaats in de gewrichten terecht komen en gedurende een lange periode aanwezig zijn. Met behulp van "gentherapie" (het behandelen van cellen met een therapeutisch gen) is het mogelijk om om specifieke cellen die betrokken zijn bij de ontwikkeling van gewrichtsdestructie ter plaatse zodanig genetisch te veranderen, dat ze zelf enzymremmers gaan produceren. Ze worden als het ware plaatselijke "medicijnfabriekjes". Een gen is een stukje stukje DNA dat de genetische informatie bevat voor een bepaald eiwit, bijvoorbeeld een enzym of een enzymremmer. Er zijn verschillende manieren om cellen therapeutische genen toe te dienen. In het kader van dit proefschrift is gebruik gemaakt van virussen. Om zich te kunnen vermeerderen infecteren virussen lichaamscellen, wat niets anders is dan dat ze hun eigen DNA in een lichaamscel brengen (Figuur 2). Van deze eigenschap wordt gebruik gemaakt om cellen een therapeutisch gen toe te dienen. Van de virussen die hier gebruikt zijn is het DNA van het virus dat nodig is om zich te vermeerderen vervangen door een stukje DNA (gen) dat de genetische informatie bevat voor een bepaalde enzymremmer. Een dergelijk genetisch veranderd virus is onschadelijk, maar is nog wel in staat een cel te infecteren. Het nieuwe gen in de cel zorgt ervoor dat de cel de enzymremmer gaat maken en uitscheiden (Figuur 2). Een van de voordelen van gentherapie is dat cellen gedurende een lange periode hun eigen medicijn kunnen maken. Bovendien kan door bepaalde cellen als doelwit te nemen, de productie van remmers beperkt blijven tot een klein gebied, waardoor de kans op bijwerkingen op andere plaatsen in het

lichaam veel kleiner wordt. In de hoofdstukken 4 en 6 worden de effecten van gentherapie met enzymremmers beschreven.

De reumatoïde synoviale fibroblast

Omdat er van een bepaalde cel in het synovium, de synoviale fibroblast, anwijzingen zijn dat deze een cruciale rol speelt in het ontstaan van gewrichtsschade bij reuma, staat deze cel centraal in de onderzoeken die beschreven worden in de hoofdstukken 2, 4 en 6 van dit proefschrift. Deze reumatoïde synoviale fibroblast is in staat om het kraakbeen te beschadigen door kraakbeenbestandelen afte breken en in het kraakbeen te groeien. Dit is vergelijkbaar met het ingroeien van een kankercel in eromheen liggend weefsel. Dit proces waarbij cellen in weefsels groeien waar ze niet horen wordt "invasie" genoemd. We hebben de invasie van reumatoïde synoviale fibroblasten onderzocht in kweekmodellen en in een diermodel. In het diermodel worden reumatoïde synoviale fibroblasten, die nadat ze bij een operatie uit een reumapatiënt zijn gehaald enige tijd zijn gekweekt, samen met een stukje menselijk kraakbeen onder het nierkapsel van een muis geïmplanteerd (Figuur 3). De muizen die voor dit experiment gebruikt worden, zogenaamde *severe combined immunodeficient* (SCID) muizen, hebben een niet goed functionerend immuunsysteem en stoten daarom de implantaten niet af. Na 60 dagen worden het kraakbeen en de reumatoïde synoviale fibroblasten uit de muizen gehaald. Bij microscopisch onderzoek blijkt dat de reumatoïde synoviale fibroblasten in het kraakbeen zijn gegroeid en het kraakbeen voor een deel hebben afgebroken. Omdat dit lijkt op de kraakbeeninvasie in een gewricht van een reumapatiënt, wordt dit SCID muis co-implantatie model als een bruikbaar model voor kraakbeendestructie bij reuma beschouwd.



Figuur 3. SCID muis co-implantatie model. *Gekweekte synoviale cellen worden geïnfecteerd met genetisch gemodficeerde virusdeeltjes en vervolgens met een stukje menselijk kraakbeen onder het nierkapsel van een SCID muis geïmplanteerd. Na 60 dagen worden het kraakbeen en de cellen eruit gehaald en wordt onder de microscoop de mate van kraakbeeninvasie door de synoviale cellen geëvalueerd.*

Samenvatting van de resultaten van dit proefschrift

Omdat nog niet geheel duidelijk is welke factoren er toe bijdragen dat reumatoïde synoviale fibroblasten in het kraakbeen groeien, hebben we onderzocht of kraakbeencellen (chondrocyten) invloed uitoefenen op het gedrag van de reumatoïde synoviale fibroblasten. De resultaten, beschreven in **Hoofdstuk 2**, duiden erop dat chondrocyten het invasieve gedrag van reumatoïde synoviale fibroblasten inderdaad kunnen beïnvloeden. Uit een experiment in het SCID-muis co-implantatiemodel bleek dat de mate van invasie van reumatoïde synoviale fibroblasten minder was in kraakbeen dat een 1-2 dagen bewaard was alvorens het gebruikt werd dan in kraakbeen dat direct nadat het uit een patient was gekomen gebruikt werd. Deze observatie is in een kweekmodel voor kraakbeendestructie verder onderzocht. Het blokkeren van de eiwitproductie van de chondrocyten in dit model had tot gevolg dat reumatoïde synoviale fibroblasten minder goed kraakbeen af konden breken. Dit wijst erop dat eiwitten die de chondrocyten uitscheiden de reumatoïde synoviale fibroblasten stimuleren tot het het afbreken van de kraakbeenmatrix. Verder onderzoek wees uit dat het eiwit interleukine-1-β (IL-1-β) de reumatoïde synoviale fibroblasten kan stimuleren tot de afbraak van kraakbeenmatrix. IL-1-β is een eiwit dat een rol speelt in het immuunsysteem (cytokine) en een ontstekingsbevorderend effect heeft. Daarnaast kan IL-1-β cellen stimuleren meer proteolytische enzymen te produceren. De resultaten van Hoofdstuk 2 wijzen erop dat de invloed van chondrocyten op reumatoïde synoviale fibroblasten o.a. zou kunnen werken via de uitscheiding van IL-1-β.

In de hoofstukken 3-7 is de rol van verschillende proteolytische enzymen bij het ontstaan van gewrichtsdestructie bij reuma onderzocht en is gekeken naar manieren om deze te kunnen remmen. In **Hoofdstuk 3** is onderzocht of het proteolytische enzym granzym B een rol zou kunnen spelen bij de afbraak van gewrichtskraakbeen in reumapatiënten. Granzymen worden gemaakt door bepaalde witte bloedcellen en spelen een rol in de afweer, doordat ze cellen die bijvoorbeeld door een virus geïnfecteerd zijn aan kunnen zetten tot celdood. Uit eerder onderzoek is gebleken dat granzym producerende cellen in verhoogde mate aanwezig zijn in het synovium van aangedane gewrichten van reumapatiënten. Uit de resultaten van Hoofdstuk 3 blijkt dat granzym B direct in staat is om een van de belangrijkste kraakbeen bestanddelen, de proteoglycanen, af te breken. Bovendien blijkt dat granzym B aanwezig is in de buurt van de plaats waar de kraakbeen- en botbeschadigingen ontstaan. Deze resultaten duiden erop dat granzym B een rol zou kunnen spelen bij het ontstaan van gewrichtsdestructie bij reuma. Verder onderzoek zal moeten uitwijzen hoe belangrijk granzym B is bij het ontstaan van gewrichtsbeschadiging bij reumapatiënten.

In **Hoofdstuk 4** staat het proteolytische enzym plasmine centraal. De meest bekende functie van plasmine is de afbraak van bloedstolsels in de bloedsomloop. Daarnaast speelt plasmine een belangrijke rol bij processen waarbij weefsel afgebroken wordt. Als plasmine zijn werk

doet bij weefselafbraak, zit het meestal gebonden aan het oppervlak van cellen. Ongebonden plasmine wordt namelijk vrijwel direct geremd door allerlei remmers. Plasmine komt in het gehele lichaam voor als inactief pro-enzym, plasminogeen. Op die plaatsen waar plasmine nodig is om weefsel af te breken, wordt het geactiveerd door een van zijn activatoren urokinase. Uit eerder onderzoek blijkt dat urokinase in het synovium van reumapatiënten in verhoogde mate voorkomt. De resultaten beschreven in Hoofdstuk 4 laten zien dat plasmine in staat is in een kweekmodel een kraakbeen-achtige matrix af te breken. Een overmaat van een plasmineremmer is in staat om die afbraak compleet te remmen. Dit wijst erop dat plasmine een rol zou kunnen spelen bij de destructie van kraakbeen bij reuma. Het remmen van plasmine zou daarom een zinnige manier kunnen zijn om kraakbeendestructie bij reuma te remmen. Om op een efficiënte manier plasmine aan het celoppervlak te remmen is een remmer ontworpen die bindt aan het celoppervlak en ter plaatse plasmine remt. De effecten van deze celgebonden plasmineremmer zijn onderzocht in modellen voor kraakbeenafbraak en kraakbeeninvasie. Via gentherapie (zie boven) zijn reumatoïde synoviale fibroblasten dusdanig genetisch veranderd dat ze deze celgebonden plasmineremmer in grote hoeveelheden uitscheiden. De celgebonden plasmineremmer blijkt op effectieve wijze kraakbeenafbraak door plasmine te kunnen remmen, veel effectiever dan een plasmine remmer die niet aan het celoppervlak bindt. Deze resultaten wijzen erop dat plasmine inderdaad een belangrijke rol zou kunnen spelen bij de afbraak van kraakbeen door reumatoïde synoviale fibroblasten en dat dit effectief geremd kan worden met een celgebonden plasmineremmer.

Om het effect van plasmineremming in reumapatiënten te onderzoeken is in **Hoofdstuk 5** een bestaand medicijn gebruikt dat plasmine-activiteit remt. Dit medicijn, tranexaminezuur (Cyklokapron®), wordt al jaren gebruikt om bloedingen te voorkomen in patiënten die bijvoorbeeld net geopereerd zijn en heeft slechts lichte bijwerkingen. In eerdere onderzoek in proefdieren en reumapatiënten zijn aanwijzingen gevonden dat tranexaminezuur een gewrichtssparende werking zou kunnen hebben. In een dubbelblind, placebo gecontrolleerd onderzoek, hebben wij de effectiviteit van tranexaminezuur verder onderzocht. In een dubbelblind, placebo gecontrolleerd onderzoek werden de patiënten die hebben meegedaan aan het onderzoek verdeeld in twee groepen, waarvan de ene groep tranexaminezuur heeft gekregen en de andere groep het nepmedicijn (placebo), zonder dat de patiënten of de onderzoeker wisten wat ze geslikt hadden. Bij de patiënten die meegedaan hebben aan dit onderzoek was de activiteit van de ziekte redelijk onder controle. Desalniettemin werden in verhoogde mate afbraakproducten van kraakbeen en bot in de urine gevonden. Dit wijst erop dat in deze patiënten, ondanks de onderdrukte ziekteactiviteit, het proces van gewrichtsdestructie toch nog doorging. De resultaten, die beschreven staan in Hoofdstuk 5, hebben geen gunstig effect van tranexaminezuur kunnen laten zien op de hoeveelheid afbraak producten van kraakbeen en bot in de urine. Hieruit kan geconcludeerd worden dat behandeling met tranexaminezuur geen toegevoegde waarde heeft als gewrichtssparend

medicijn, tenminste niet bij reumapatiënten bij wie de ziekte redelijk onder controle is. Of tranexaminezuur bij patiënten met een ernstige opvlammende reuma wel effectief is, kunnen we op basis van de resultaten van dit onderzoek niet zeggen.

Omdat bij het ontstaan van beschadigingen aan het kraakbeen en bot in de gewrichten ook een belangrijke rol aan een andere familie van enzymen, de MMPs, wordt toegedicht, is in **Hoofdstuk 6** de rol van MMPs bij kraakbeendestructie bestudeerd en is onderzocht of met behulp van gentherapie met MMP-remmers kraakbeendestructie geremd kon worden. De MMPs vormen een grote familie van ten minste 20 enzymen, die samen in staat zijn ongeveer alle typen eiwitten die in de weefsels voorkomen af te breken. Dat maakt MMPs in potentie gevaarlijke enzymen. Een overmaat aan remmers in de weefsels, de ***t**issue **i**nhibitors of **m**etallo**p**roteinases* (TIMPs), houden de MMPs in bedwang. Doordat er in het synovium van een door reuma aangedaan gewricht veel meer MMPs worden uitgescheiden, is er een tekort aan remmers om de schadelijke activiteiten van de MMPs te blokkeren. Daarom zou behandeling met MMP remmers een manier kunnen zijn om gewrichtsdestructie door MMPs te remmen. De benadering die in hoofdstuk 6 is gekozen is om door middel van gentherapie via een genetisch aangepast virus de reumatoïde synoviale fibroblasten een overmaat aan TIMPs te laten produceren. Er zijn vier verschillende TIMPs bekend, TIMP-1, -2, -3 en -4. In dit onderzoek zijn de effecten van TIMP-1 en TIMP-3 op de invasie van kraakbeen door reumatoïde synoviale fibroblasten onderzocht. TIMP-1 en TIMP-3 lijken voor wat betreft hun remmende werking erg op elkaar, maar zij verschillen in een paar aspecten. TIMP-1 is een oplosbaar eiwit, terwijl TIMP-3 aan eiwitten in de matrix tussen de cellen (extracellulaire matrix) blijft plakken. Daarnaast remt TIMP-3 bepaalde MMPs die aan het celoppervlak vastzitten, de zogenaamde ***m**embrane-**t**ype* (MT) MMPs, terwijl TIMP-1 dat niet kan. Bovendien heeft TIMP-3 ook nog andere eigenschappen. Het remt bijvoorbeeld ook het vrijkomen van bepaalde celgebonden eiwitten die daardoor in hun functie belemmerd worden. De resultaten in Hoofdstuk 6 laten zien dat de invasie van kraakbeen door de reumatoïde synoviale fibroblasten effectief geremd kan worden door gentherapie met zowel TIMP-1 en TIMP-3. In een kweekmodel van kraakbeeninvasie bleek TIMP-3 beter te remmen dan TIMP-1. Dit wijst erop dat de specifieke eigenschappen van TIMP-3 een toegevoegde waarde zouden kunnen hebben voor de remming van het invasieve gedrag van de reumatoïde synoviale fibroblasten. De eigenschap dat TIMP-3 aan extracellulaire matrixeiwitten blijft plakken zou gunstig kunnen zijn, omdat daardoor TIMP-3 in de buurt van de cellen blijft. Daarnaast zou het sterkere effect erop kunnen duiden dat de celgebonden MT-MMPs belangrijk zijn bij het proces van kraakbeeninvasie. Verder bleek TIMP-3 in sterkere mate dan TIMP-1 de groeisnelheid van reumatoïde synoviale fibroblasten te kunnen remmen. De conclusies die uit deze resultaten getrokken kunnen worden zijn dat MMPs een belangrijke rol lijken te spelen bij de invasie van kraakbeen door reumatoïde synoviale fibroblasten en dat invasie effectief geremd kan worden door gentherapie met TIMP-1, en met name door TIMP-3.

Omdat MMPs belangrijk lijken te zijn bij het ontstaan van gewrichtsdestructie bij reumapatiënten, zou een therapeutische benadering met een MMP-remmer het ontstaan of het voortschrijden van gewrichtsdestructie moeten kunnen remmen. Doxycycline is een veel gebruikt antibioticum dat ook in staat is MMPs te remmen. Eerder onderzoek heeft laten zien dat doxycycline mogelijk een gewrichtssparende werking heeft. Dit onderzoek was echter niet uitgevoerd met een placebo groep. Daarom hebben wij in een dubbelblind, placebo gecontroleerd onderzoek de effecten van doxycycline bij reumapatiënten onderzocht. De resultaten van dit onderzoek, beschreven in **Hoofdstuk 7**, laten zien dat doxycycline geen enkel effect heeft op de progressie van gewrichtsdestructie, gemeten door afbraakproducten van kraakbeen en bot in urine en afgelezen op röntgenfoto's. Doxycycline lijkt dus niet een geschikt middel om gewrichtsdestructie te remmen bij reumapatiënten. Mogelijkerwijs remt doxycycline niet de cruciale MMPs of is er een veel hogere dosis nodig om een effect te bewerkstelligen. Om proteolytische enzymen die gewrichtsdestructie veroorzaken op een effectieve manier te remmen zal dus naar andere methoden gezocht moeten worden.

Conclusies

Uit de onderzoeken die in dit proefschrift beschreven zijn is naar voren gekomen dat plasmine, MMPs en mogelijk ook granzym B een rol spelen bij de ontwikkeling van gewrichtsdestructie bij patiënten met reuma. Met reeds bestaande geneesmiddelen die proteolytische enzymen kunnen remmen, zoals tranexaminezuur en doxycycline, die als pillen worden toegediend, blijkt het niet mogelijk om de activiteit van de schadelijke enzymen in de gewrichten effectief te remmen. Resultaten in kweekmodellen en in het SCID muis co-implantiemodel laten zien dat door enzymremmers naar de juiste plaats te brengen, bijvoorbeeld met behulp van gentherapie en door remmers te gebruiken die aan de juiste cellen bijven plakken, mogelijk wèl effectieve remming bewerkstelligd kan worden. Voortzetting van onderzoek naar deze nieuwe remmers kan bijdragen tot de ontwikkeling van nieuwe behandelstrategieën voor reumapatiënten die specifiek de gewrichtsdestructie bij deze patiënten kunnen voorkomen.

LIST OF ABBREVIATIONS

Ad	Adenovirus
ATF	Amino terminal fragment
ATF.BPTI	Fusion protein of ATF and BPTI
BPTI	Bovine pancreatic trypsine inhibitor
CRP	C-reactive protein
DAS	Disease activity scale
DMARD	Disease modifying anti-rheumatic drug
DMEM	Dulbecco's modified eagle's medium
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ESR	Erythrocyte sedimentation rate
FCS	Fetal calf serum
GAG	glycosaminoglycan
^{3}H	Radio-labeled hydrogen
HP	hydroxylysylpyridinoline
HPLC	High-performance liquid chromatography
IL	Interleukin
Ig	Immuneglobuline
IU	International unit
KIU	Kallekrein inhibiting unit
LP	lysylpyridinoline
MCP	Metacarpophalangeal (joint)
MMP	Matrix metalloproteinase
MT-MMP	Membrane-type MMP
NCS	Newborn calfserum
NHS	Normal human serum
NSAID	Non-steroidal anti-inflammatory drug
OA	osteoarthritis
PA	Plasminogen activator
PAI	Plasminogen activator inhibitor
PCR	Polymeric chain reaction
PFU	Plaque forming unit
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RT-PCR	Reversed transcriptase-PCR
^{35}S	Radio-labeled sulfate
SCID	Severe combined immunodeficient
SEM	Standard error of the mean
TACE	TNF-α converting enzyme
TIMP	Tissue inhibitor of metalloproteinases
TNF	Tumor necrosis factor
tPA	Tissue-type plasminogen activator
uPA	Urokinase-type plasminogen activator
uPAR	UPA receptor
VAS	Visual analogue scale

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CURRICULUM VITAE

Willemijn Henrieke van der Laan werd geboren op 28 mei 1970 te Rotterdam. Na het behalen van het VWO diploma aan het Gymnasium Erasmianum te Rotterdam in 1988, begon zij in 1989 aan de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam. Tijdens deze studie werd medisch psychologisch onderzoek verricht in het Crawford Long Hospital te Atlanta in de Verenigde Staten van Amerika. Hierin werd zij begeleid door Prof. Dr. P.S. Sebel, als anaesthesioloog verbonden aan Emory University te Atlanta, en Dr. B. Bonke, als psycholoog verbonden aan de Erasmus Universiteit te Rotterdam. In januari 1997 slaagde zij voor het artsexamen.

Vanaf april 1997 tot april 2001 verrichtte zij als klinisch onderzoeker wetenschappelijk onderzoek in het Gaubius Laboratorium, TNO Preventie en Gezondheid, binnen de afdeling Vaat- en Bindweefselonderzoek en in het Leids Universitair Medisch Centrum binnen de afdeling Reumatologie. De resultaten van dit onderzoek staan beschreven in dit proefschrift. Zij werd begeleid door Dr. J.H. Verheijen, verbonden aan het Gaubius Laboratorium, en Prof. dr. T.W.J. Huizinga, verbonden aan de afdeling Reumatologie van het Leids Universitair Medisch Centrum.

Vanaf oktober 2001 is zij gestart met de opleiding tot internist/reumatoloog van het Academisch Medisch Centrum te Amsterdam. Zij is heden werkzaam als arts-assistent geneeskunde in opleiding in het Kennemer Gasthuis te Haarlem.

NAWOORD

Er zijn vele mensen die bijgedragen hebben aan de totstandkoming van dit proefschrift. De collega's van de divisie Vaat- en Bindweefselonderzoek van TNO Preventie en Gezondheid ben ik bijzonder dankbaar, omdat zij mij de kunst van experimenteel laboratoriumonderzoek hebben geleerd. De prettige werksfeer en bereidheid van om elkaar te helpen hebben de uitvoering van dit promotieonderzoek gemakkelijker en plezierig gemaakt. Mijn directe collega's wil ik bedanken voor de nuttige tips tijdens de werkbesprekingen en de gezelligheid op en buiten de werkvloer. De collega's in de "Endotheelgang" wil ik bedanken voor hun hulp bij de experimenten die ik aldaar heb uitgevoerd. De collega's van de afdeling Reumatologie van het Leids Universitair Medisch Centrum ben ik dankbaar voor hun klinische blik op mijn onderzoek. *I would like to thank the research fellows and technicians of the Centre of Experimental Rheumatology in Zürich for the very pleasant working visits.*

Met name wil ik noemen: Karel Ronday, die mij geïnspireerd heeft tot het doen van dit onderzoek; Jeroen de Roos, die mij de beginselen van celkweek heeft geleerd; Linda Huisman, die mij met raad en daad heeft bijgestaan tijdens de uitvoering van dit promotieonderzoek; Jos Grimbergen voor zijn enthousiasme, geduld en hulpvaardigheid; Elsbet Pieterman voor de plezierige samenwerking; Nico Sakkee voor het HPLC-werk en allerhande praktische tips; Ruud Bank van wiens kennis over collageen ik dankbaar gebruik heb gemaakt; Roeland Hanemaaijer voor het leveren van goede ideeën; Paul Quax voor zijn bijdragen aan de gentherapie hoofdstukken; Ellen Schenk voor de steun en gezelligheid toen wij een kamer deelden en Martine Lamfers voor het kunnen delen van "promotiestress", *I am grateful to Steffen and Renate Gay for their support and hospitality in Zürich and to Thomas Pap for his inspiration and our fruitful collaboration.*

Eelco Visser ben ik dankbaar voor zijn steun tijdens het eerste jaar van dit onderzoek. Mijn familie, vriendinnen en vrienden wil ik bedanken, omdat ze er altijd voor me zijn geweest tijdens de *ups* en *downs* van de onderzoeksperiode. *Last but not least*, noem ik Henk, die ik eindeloos dankbaar ben voor zijn liefde, begrip, relativerende vermogen, steun, goede zorgen en niet aflatende vertrouwen in mij.