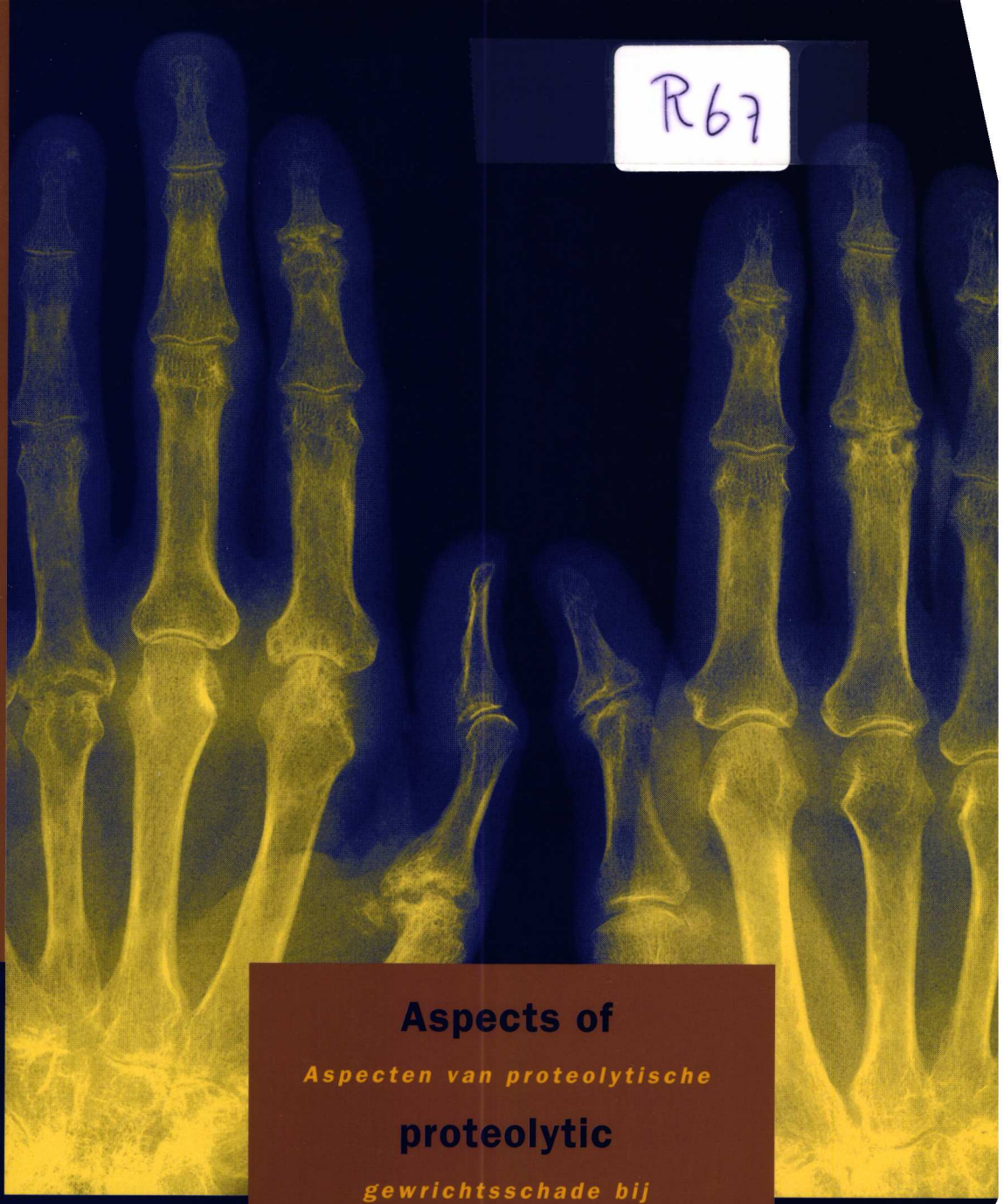

Aspects of proteolytic joint destruction in Rheumatoid Arthritis

Aspecten van proteolytische gewrichtsschade bij Reumatoïde Artritis

H. K. RONDAY

STELLINGEN

1. De verhoogde expressie in het reumatoïde synovium en het effect op bot- en kraakbeenafbraak *in vitro,* maken het plasminogeen-activeringssysteem tot een handvat voor de ontwikkeling van gewrichtsbeschermende therapie in reumatoïde artritis.
(dit proefschrift)

2. Binding van urokinase-type plasminogeen activator aan zijn celgebonden receptor heeft functionele betekenis bij de afbraak van botmatrix.
(dit proefschrift)

3. Het remmend effect van urokinase-receptor geassocieerd aprotinine op de gewrichtskraakbeenafbraak door reumatoïde synoviale fibroblasten *in vitro* geeft aan dat deze cellen, het plasminogeen-activeringssysteem gebruiken om kraakbeen af te breken.
(dit proefschrift)

4 De beschikbaarheid en de relatieve veiligheid van tranexaminezuur maken dit middel geschikt om de behandelstrategie gericht op remming van enzymatische weefselafbraak bij reumatoïde artritis te bestuderen.
(dit proefschrift)

5. De aanwezigheid van Granzyme B-houdende cellen in de chondro-synoviale overgang en het vermogen van Granzyme B tot afbraak van gewrichtskraakbeen *in vitro,* wijzen op een pathogene rol van dit enzym bij het ontstaan van gewrichtsschade in artritis.
(dit proefschrift)

6. De verlaging van synoviale metalloproteinaseactiviteit en vermindering van kraakbeenschade bij artrotische proefdieren die zijn behandeld met tranexaminezuur, illustreren de functionele relatie tussen metalloproteinasen en het plasminogeen-activeringssysteem.
(Vignon et al., J Rheumatol 1991;18:131-133)

7. De hoge synoviale trombineconcentratie bij patiënten met reumatoïde artritis en het vermogen van dit enzym om proteoglycaanafbraakprodukten vrij te maken uit gewrichtskraakbeen suggereren een rol van trombine bij het ontstaan van kraakbeenschade in reumatoïde artritis.
(Furmaniak-Kazmierczak et al., J Clin Invest 1994;94:472-480)

8. Amyloïdose moet worden overwogen als oorzaak van pijn en zwelling van meerdere gewrichten wanneer dit niet reageert op ontstekingsremmende therapie.

9. De co-incidentie van hepatitis C virus infectie en mixed cryoglobulinaemie, en het gunstig effect van antivirale therapie op het klinisch beeld van cryoglobulinaemie, suggereren een causaal verband tussen deze twee aandoeningen.
(Mertens, Ronday et al., Neth J Med 1998;51:195-250)

11. De effectiviteit van antibiotische therapie in combinatie met chirurgische drainage of met drainage per punctie bij de behandeling van septische artritis is niet wezenlijk verschillend.

12. Indien geldgebrek de verklaring blijft voor wachttijden in de gezondheidszorg, dient steun van het bedrijfsleven te worden geaccepteerd.

13. Verbetering van doelmatigheid in de gezondheidszorg wordt belemmerd door verschillende interpretatie van het begrip doelmatigheid.

14. In de wetenschap wordt de statistiek nogal eens gebruikt als een lantarenpaal door een dronkaard; voor ondersteuning en niet voor verlichting.

15. Promoveren is vaak een weg naar niets en het gaan naar nergens totdat je het bereikt hebt.
(ontleend aan Winny the Pooh)

Stellingen behorende bij het proefschrift *"Aspects of proteolytic joint destruction in rheumatoid arthritis"*

H.K. Ronday Leiden, 27 mei 1998

ASPECTS OF
PROTEOLYTIC JOINT DESTRUCTION
IN RHEUMATOID ARTHRITIS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE RIJKSUNIVERSITEIT TE LEIDEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS DR. W.A. WAGENAAR,
HOOGLERAAR IN DE FACULTEIT DER SOCIALE WETENSCHAPPEN,
VOLGENS BESLUIT VAN HET COLLEGE VOOR PROMOTIES
TE VERDEDIGEN OP WOENSDAG 27 MEI 1998
TE KLOKKE 14.15 UUR

DOOR

HERMAN KAREL RONDAIJ

GEBOREN TE MONSTER IN 1960

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Aan Marjolein

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a systemic chronic inflammatory disease that specifically targets the joint capsule (synovial tissue) of diarthrodal joints (1). The disease is characterized by joint pain, swelling and destruction of bone and cartilage, leading to functional loss and impairment of quality of life.
The overall prevalence rate for RA in western countries is about 1% with a female to male ratio of 3:1 and a peak age of onset between the fifth and sixth decade of life.
Annual incidence rates vary from 90 to 300 new cases per 100 000 (2).
RA has a wide clinical spectrum. Many patients present with non-specific systemic features associated with the onset of tenderness, stiffness and synovial swelling of one or more joints, characteristically that of the hands, wrists and feet in a symmetrical fashion.
The disease is not confined to the joints and may involve many other body systems.
The most common extra-articular features are anaemia, nodules, serositis, eye involvement, vasculitis, Sjögren's syndrome and Felty's syndrome.
The cause of RA remains unknown despite many years of research, and it is generally believed to be multifactorial. Current thinking favors the concept that environmental factors initiate an autoimmune disorder in genetically susceptible individuals, which progresses to inflammation and destruction. Hormonal factors are involved as appears from the observation that women are affected approximately three times more often than men (1) and from the protective effect of oral contraceptives (3) and pregnancy (4).
Within the joint of patients with RA, (active-) chronic inflammation of the joint capsule is the predominant mechanism leading to clinical signs and tissue destruction. Under normal conditions, the synovium is a vascular fibrous or adipose tissue covered by a layer of specialized cells (synoviocytes) that has nutrient and lubricating functions. The rheumatoid synovium, however, is a hypertrophic and hyperplastic tissue containing a mixed inflammatory cell infiltrate, endothelial and synovial cells. This cell mass produces pro-inflammatory cytokines and tissue degrading proteolytic enzymes leading to a state of tissue destruction and reparative fibrosis (figure 1).
Knowledge of the disease at the cellular and molecular level has accelerated rapidly during the past decades to a point where it is possible to describe in detail the immuno- and histopathological phenomena. The pathophysiology, however, is still incompletely understood. T cell dependent pathways are believed to play a crucial role in the initiation and perhaps perpetuation of RA. New experimental approaches during the past several years suggest a significant role of T cell independent pathways during the chronic phase of the disease (5,6). Macrophage-like type A synoviocytes and fibroblast-like type B synoviocytes account for the majority of cells in the synovial lining layer, which is the interface between synovium and the intra-articular space or the adjacent bone and cartilage (7). The former are derived from monocyte precursors in the bone marrow. Type B cells have the morphologic appearance of fibroblasts. Both cell types produce an impressive

range of cytokines and arachidonic acid metabolites, and are able to invade and destroy cartilage and bone by production of matrix degrading enzymes (5,8-10). Destruction of the extracellular matrix by synovial lining cells could result either from a response to inflammatory cytokines produced in an immune response or from a "tumor-like" transformation of synoviocytes. RA fibroblast-like synoviocytes have been shown to maintain their destructive behaviour over longer periods of time in the absence of T cells and cytokines (11,12). The low levels of T cell cytokines in established disease (13) and the abundance of lining cells has led to the theory that chronic RA is perpetuated by transformed lining cells.

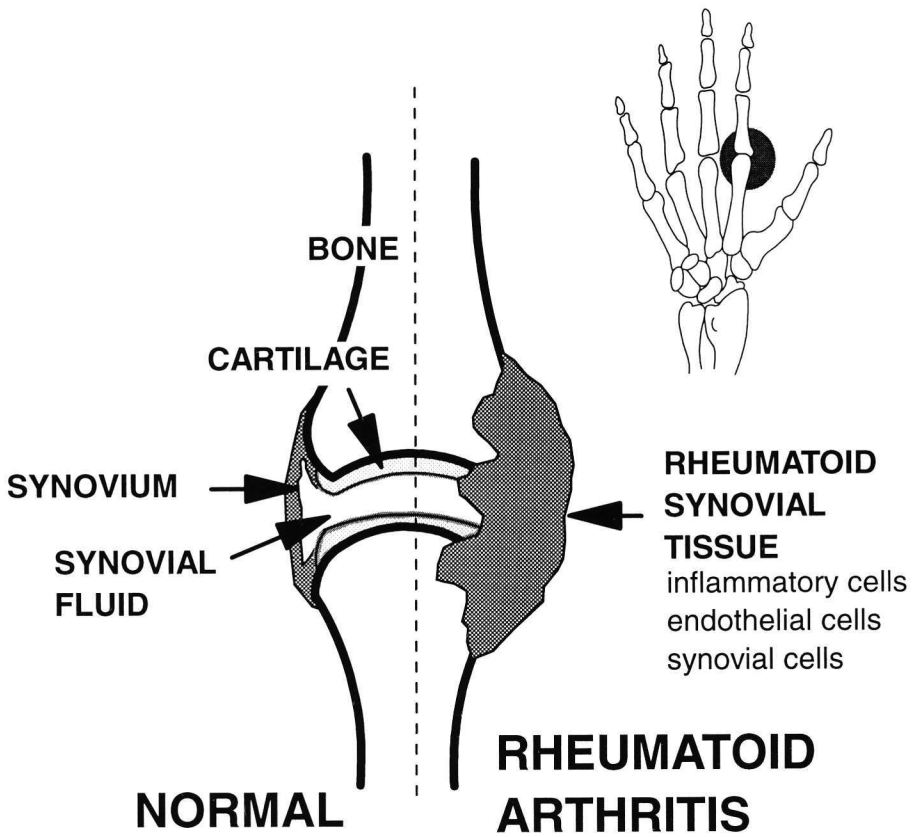

Figure 1. Schematic representation of a normal (left panel) and a rheumatoid joint (right panel). In rheumatoid arthritis, the hyperplastic and hypertrophied synovial tissue overgrows the cartilage and invades the underlying bone.

CONNECTIVE TISSUE MATRIX

Destruction of connective tissue is a characteristic consequence of RA. Connective tissue is defined as the compartment and components that provide the structural support of the body and bind together its cells, organs and tissues. The major connective tissues are skin, bone, cartilage, tendons and ligaments. The extracellular matrix of connective tissue comprises all fibers and the groundsubstance that surround individual cells. This matrix is composed of collagens, proteoglycans, elastin, fibronectin and other extracellular proteins. Collagen is the major protein of connective tissue and is the most common protein of the vertebrate body. More than 15 different kinds of collagen have been identified in different tissues of vertebrates (14). This family of collagens can be divided into four subclasses: fibrillar collagens, basement membrane-associated collagens, fibril-associated collagens, and short chain collagens. The fibrillar collagens, type I, II, and III, are the major structural constituents of bone, cartilage, ligaments, tendons and skin. Types IX and XI collagens (fibril-associated) also occur in cartilage. The distribution of the various collagen types has been reviewed by Williams et al. (15). Fibrillar collagen has an unique triple helical structure (14). This conformation is the result of the winding of three separate polypeptide chains (α chains, each containing approximately 1000 amino acid residues) wrapped around one another in a higher order rope-like fashion to produce the triple helical structure of the collagen molecule. At the N-terminal and C-terminal ends, these intertwined chains have non-helical peptide extensions called telopeptides.
The conformation of the triple helix is stabilized by interchain hydrogen bonds. Folding of the α chains into a triple helix requires glycine to be present as every third amino acid residue. The α chains therefore are composed of series of triplet Gly-X-Y sequences in which frequently X is proline and Y is hydroxyproline. Newly synthesized procollagen molecules are secreted into the extracellular matrix where they are processed by enzymes to undergo fibril formation. These enzymes are procollagen N-proteinase (removal of N-propeptide), procollagen C-proteinase (removal of C-propeptide) and lysyl oxidase (initiation of crosslinking). The molecules are assembled into staggered arranged fibrils with crosslinks between the molecules to stabilize the structure and to give the connective tissue its strength and rigidity (16) (figure 2).

The other major proteins found in connective tissue are the proteoglycans (PGs). PGs are complex macromolecules consisting of a polypeptide chain (core protein) which contains three globular domains (G1-G3), interspersed by an extended linear region with covalently bound glycosaminoglycan (GAG) chains (17). GAGs are linear polymers of repeating disaccharides that contain one hexosamine and either a carboxylate or a sulfate ester, or both.

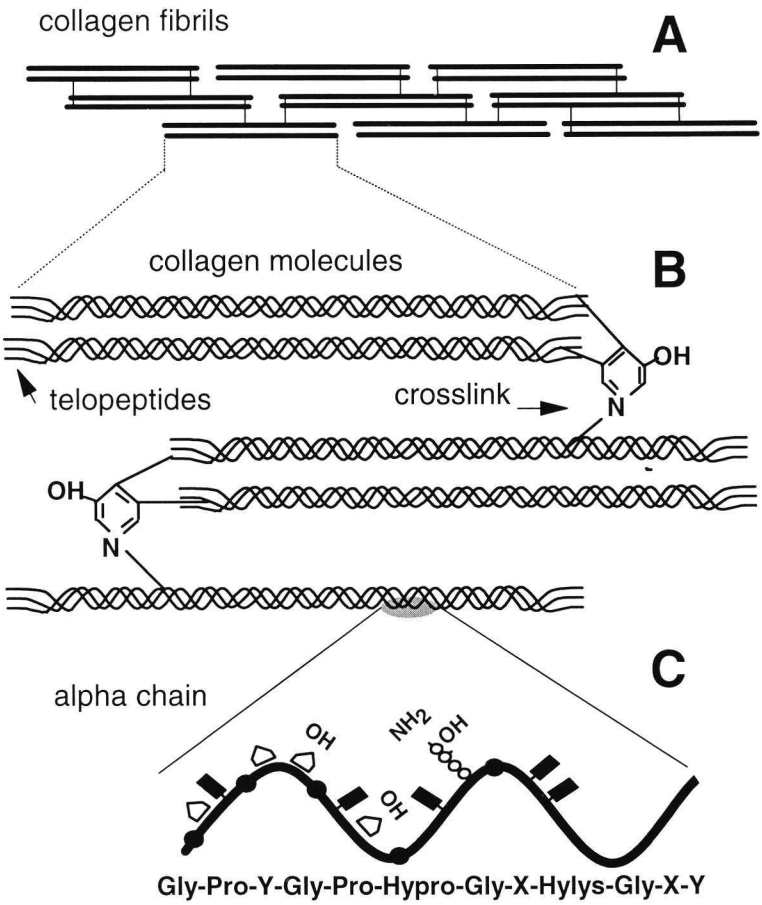

Figure 2. Diagram of collagen type I structure. Panel A shows the packing arrangement of collagen molecules in the microfibrils. Panel B shows the triple helix of the collagen molecules, connected by crosslinks. The individual α-chains have glycine in every third position and contain large amounts of proline and hydroxyproline (panel C)

The first globular domain (G1) is a link protein that associates the core protein with hyaluronic acid to form highly charged aggregates, which are responsible for pulling water into the tissue and creating a compression resisting swelling pressure (figure 3).

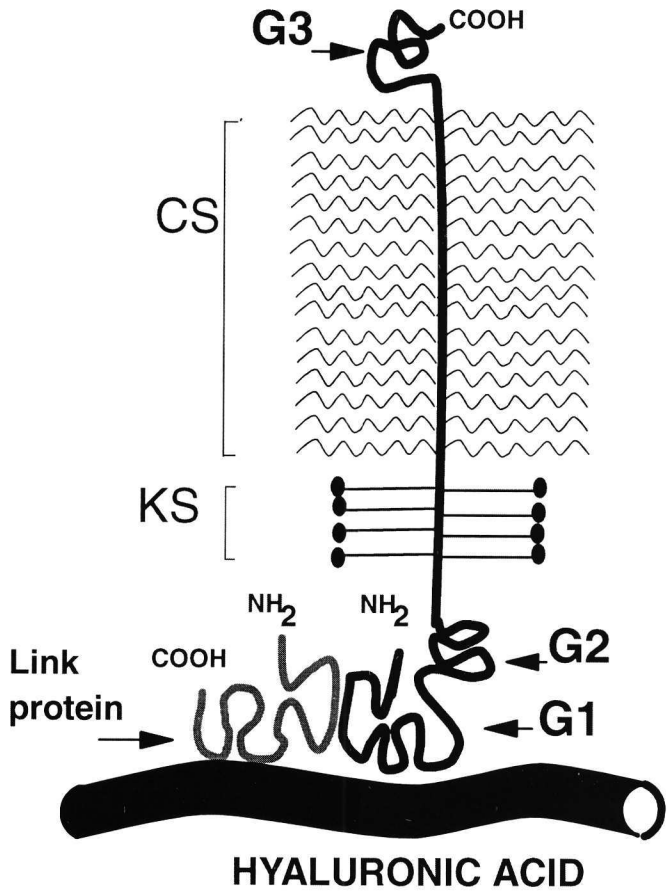

Figure 3. Diagram of the various components of the proteoglycan molecule. G= Globular domain. CS and KS are the Glycosaminoglycans: Chondroitin Sulphate and Keratan Sulphate respectively

Fibronectin is an adhesive glycoprotein that plays a role in mediating cell-cell as well as cell-matrix interactions. Elastin is a polymer whose structure and crosslinking gives it elastic properties which is necessary in distensible tissues such as lung parenchyma, arterial cell walls and skin. Recently, immunoassays and chemically-based assays have been developed making it possible to detect the release of connective tissue derived products into body fluids such as synovial fluid, serum, and urine. There is great interest in the use of biochemical markers to monitor or predict the progress of joint destruction or

follow the efficacy of therapeutic intervention in individual patients suffering from RA. These markers include released fragments of matrix components and enzymes (and their inhibitors) involved in bone and cartilage breakdown. In *in vitro* studies the release of hydroxyproline, and radiolabeled proline and sulphate are often used to monitor the degradation of collagen and proteoglycans of matrix and tissue explants.
Degradation of collagen *in vivo* can be followed by the release of hydroxyproline and of the collagen crosslinks pyridinoline (hydroxylysylpyridinoline; HP) and deoxypyridinoline (lysylpyridinoline; LP) into serum and by their excretion in urine. HP is abundantly present in cartilage and connective tissue (18). LP is mainly derived from bone collagen (19). In RA, the urinary excretion rates of HP and LP have been correlated well with global scoring of joint damage (20) as well as with parameters of joint inflammation (21). Other collagen degradation products include N-telopeptide (NTx) , type I collagen carboxy terminal peptide (ICTP), C-propeptide of type II procollagen (CPII) (22). Biological markers for monitoring the non-collagenous tissue involvement in arthritis include chondroitin sulphate 846 epitope, keratan sulphate, hyaluronic acid, cartilage oligomeric matrix protein (COMP) and bone sialoprotein (23). Serum levels of COMP, 846 epitope (24) and hyaluronic acid (25) have been reported to possess predictive significance with respect to the rate of joint destruction in patients with early RA .
These markers could offer great opportunities to provide a suitable surrogate for radiologic and/or functional status. However, much knowledge is missing concerning the exact relationship between the type of marker and the process that is reflected (degradation or synthesis). The longitudinal and cross-sectional variation of its concentration in different body fluids, its response to treatment and its relationship with age, sex, disease severity or duration make it difficult to validate markers as diagnostic, prognostic, disease severity or outcome parameters (26).

PROTEOLYTIC PATHWAYS OF EXTRACELLULAR MATRIX DEGRADATION.

RA has both inflammatory and destructive properties. Synovial fibroblasts are important effector cells able to degrade and invade extracellular matrix of surrounding tissue (5).
The term extracellular matrix degradation includes a wide spectrum of biological processes characterized by remodeling of connective tissue and cell migration. Ovulation, morphogenesis, tissue synthesis, regeneration and involution are examples of dynamic remodeling processes, mediated by a controlled machinery of proteolytic enzymes.
Under normal physiological conditions, degradation and synthesis of extracellular matrix components are precisely regulated. Specialized matrices such as bone and cartilage are dynamic structures that resorb and expand continuously in response to stimuli.
In pathological situations however, elevated production and activation of proteolytic enzymes are no longer balanced by endogenous proteinase inhibitors. This may lead to tissue destruction and subsequent functional loss. In rheumatoid arthritis, the inflamed synovium is no longer under normal control of mechanisms that inhibit synovial overgrowth. The synovium starts to encroach the cartilage and causes loss and destruction of cartilage and bone by releasing or causing the release of tissue degrading enzymes (27-29).
The proteins of extracellular matrix in connective tissue are degraded primarily by endopeptidases (proteinases) which are divided into four classes; aspartic proteinases, cysteine proteinases, serine proteinases and matrix metalloproteinases. The proteinases are classified according to the amino acid or chemical group at the active centre of the enzyme, which cleaves the peptide bond within the target protein.
The proteolytic enzymes from the four classes that are likely to participate in extracellular matrix degradation are listed in table 1.

Table 1. Proteinases that may be involved in degradation of extracellular matrix

Enzyme		Source	Inhibitor
Aspartic proteinases			
Cathepsin D		lysosome	Pepstatin, α_2-M
Cysteine proteinases			
Cathepsin B		lysosome	Cystatins, α_2-M
Cathepsin L		lysosome	Cystatins α_2-M
Cathepsin S		lysosome	Cystatins α_2-M
Calpain		cytosol	Calpastatin
Serine proteinases			
Neutrophil elastase		PMN Leukocytes	α_1-PI
Cathepsin G		PMN Leukocytes	α_1-Antichymotrypsin
Proteinase 3		PMN Leukocytes	α_1-PI, elafin
Plasmin		plasma	Aprotinin, Protease nexin-1 α_2-Antiplasmin
Plasma kallikrein		plasma	Aprotinin
Tissue kallikrein		glandular tissues	Aprotinin, kallistatin
Tissue plasminogen activator(tPA)		endothelial cells, chondrocytes	PAI-1, PAI-2
Urokinase (uPA)		fibroblasts, chondrocytes	PAI-1, PAI-2, Protease nexin-1
Thrombin		plasma	Protease nexin-1
Mast cell chymase		Mast cells	α_1-PI
Mast cell tryptase		Mast cells	Aprotinin,
Trypstatin			
Granzymes		Natural Killer cells, T-lymphocytes	-
Matrix metalloproteinases (MMPs)			
Interstitial collagenase-1	(MMP-1)	Fibroblasts, chondrocytes	α_2-M, TIMP-1,
TIMP-2			
Interstitial collagenase-2	(MMP-8)		
Interstitial collagenase-3	(MMP-13)		
Stromelysin-1	(MMP-3)	Fibroblasts, chondrocytes	α_2-M, TIMP-1,
TIMP-2			
Stromelysin-2	(MMP-10)		
Stromelysin-3	(MMP-11)		
Matrilysin	(MMP-7)	Macrophages	TIMP
Metalloelastase	(MMP-12)		
Neutrophil collagenase	(MMP-8)	PMN leukocytes	TIMP
Gelatinase A	(MMP-2)	PMN leukocytes, macrophages	TIMP
Gelatinase B	(MMP-9)	fibroblasts, chondrocytes, osteoclasts	
Membrane-Type MMP	(MT-MMP)		(ref. 29)

CARTILAGE DEGRADATION

Articular cartilage is composed of chondrocytes and extracellular matrix which is synthesized and can be degraded by these cells. Regulation of matrix synthesis and degradation is an integral requirement for maintenance of functional cartilage.
In arthritis, the normal turnover of cartilage matrix is disturbed as a result of chemical (cytokines, proteinases) and mechanical influences leading to degradation. This remodeling involves both damage to fibrillar collagen (loss of tensile properties), as well as loss of proteoglycans (loss of compressive stiffness) and inhibited synthesis of matrix components (30).
In rheumatoid arthritis there are at least two basic mechanisms underlying cartilage erosion;
a. Extrinsic cartilage degradation brought about either by synovial cells at the pannus cartilage junction or bone derived chondroclasts at subchondral sites.
b. Intrinsic chondrolysis activity, caused by chondrocytes.

Extrinsic cartilage degradation
The cells that occupy the the cartilage pannus interface include mainly macrophages and fibroblasts but also polymorphonuclear cells, mast cells and endothelial cells of small vessels (31) and multinucleated synovial giant cells (32). Degradation of cartilage matrix primarily results from the activity of proteolytic enzymes produced by these cells (33,34). The subchondral sites of bone and cartilage degradation contain multinucleated cells at the resorption sites for calcified and hyaline cartilage (35). Chondroclasts are morphologically and histochemically identical to osteoclasts. They are both bone derived cells and create a second erosive front resulting in a bidirectional attack on articular cartilage (27).

Intrinsic cartilage degradation
The finding of collagen degradation products around chondrocytes in even the deeper zones of the cartilage matrix indicates intrinsic chondrolysis (35). This chondrocyte mediated cartilage degradation results from the response of chondrocytes to proinflammatory mediators like TNF-α and β, IL-1β (10), and histamine (36).

BONE DEGRADATION

In RA, in addition to destruction of cartilage, bone is also degraded leading to generalized and periarticular bone loss, and bone erosions (37). Remodeling of bone is cyclical process that involves phases of bone resorption and replacement. Resorption of bone is carried out by multinuclear cells of hematopoietic lineage, known as osteoclasts, whereas osteoblasts are mainly responsible for deposition of new bone matrix (38). It has been hypothesized that osteoblast-derived matrix metalloproteinases (MMPs) and cysteine proteinases play an important role in bone resorption, particularly in removal of the surface osteoid that preceeds osteoclast attachment (39). Osteoclasts remove the mineral phase of underlying bone by creating an acidic environment. The exposed proteinaceous, decalcified matrix is further degraded by proteolytic enzymes produced by both osteoclasts and osteoblasts (39-42).

Local bone loss is an early feature of rheumatoid arthritis, followed, over a longer period of time, by generalized osteoporosis. Periarticular and generalized osteopenia are mainly the result of prostaglandin and cytokine mediated alterations in the bone remodeling cycle in which factors such as disuse-related bone loss are also involved (43).

The exact mechanism of bone destruction leading to subchondral bone cysts and bone erosions is still poorly understood. Locally increased levels of prostaglandins and cytokines may stimulate osteoclastic bone resorption as well as the production of tissue proteinases (43). Synovial macrophages are believed to play a crucial role in the development of bone erosions. They release numerous cytokines, principally IL-1 and TNF-α, which act on osteoclasts to stimulate bone resorption (43). In addition, they release or enhance the release of prostaglandins and matrix degrading proteinases which may play a role in bone lysis (6,44,45). Recently, Fujikawa et al. showed that synovial macrophages, like monocytes are capable of altering their phenotype to that of osteoclasts when placed in the cellular and humoral microenvironment of bone (that is, when they come in contact with bone lined by osteoblasts in the presence of 1,25 $(OH)_2$ vitamin D_3 and macrophage colony stimulating factor) (46). This macrophage-osteoclast differentiation may be important in local bone destruction by inflamed synovial tissue.

PROTEOLYSIS IN RHEUMATOID ARTHRITIS

Although all classes of proteolytic enzymes may be involved, rheumatoid joint destruction has mainly been ascribed to serine proteinases (47) and MMPs. Among the serine proteinases, plasmin and the plasminogen activation (PA) may be of specific interest. Plasmin is able to degrade extracellular proteins directly and indirectly by activation of latent forms of MMPs (48,49).
A recently discovered set of serine proteinases, called granzymes, may also be involved in joint destruction (50,51).

THE PLASMINOGEN ACTIVATION SYSTEM

plasmin/plasminogen
In general, the PA system is involved in a large number of biological phenomena and a wide variety of diseases in which migration of cells and remodeling of tissue occurs. Much insight in the relevance of the PA system in biological processes such as hemostasis and extracellular matrix degradation has been obtained from gene transfer and targeted gene manipulation studies in mice (52-54).
Plasminogen is a 90 kD glycoprotein, that is abundantly present in the vasculature and extravascular compartments. The concentration of plasminogen in serum and interstitial fluid is approximately 100-200 μg/ml (1-2 μM). The liver is probably the principal site of synthesis (55) although granulocytes and the kidney may be alternate sources (56).
Plasminogen is an inactive pro-enzyme that can be converted to active plasmin by specific plasminogen activators. After cleavage, the formed serine proteinase plasmin consists of a light chain and a heavy chain that are linked by disulfide bonds. The light chain contains the proteolytic domain. The heavy chain contains lysine-binding sites located in the five kringles which mediate association with fibrin or fibrinogen, extracellular matrix components (57) and cell surface proteins (58). Tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) appear to be the major physiological activators of plasminogen.
Like PAs, plasmin belongs to the serine proteinase family and has tryptic specificity. Although plasmin is best known for its fibrinolytic activity, it also has the capacity to degrade many extracellular proteins such as proteoglycans, gelatins, fibronectin and laminin. In addition, it is one of the activators of pro-u-PA and of pro-MMPs (49,59).

plasminogen activators
Plasminogen activators (PAs) are highly specific serine proteinases which catalyze the conversion of plasminogen into active plasmin (60). Two structurally, immunologically

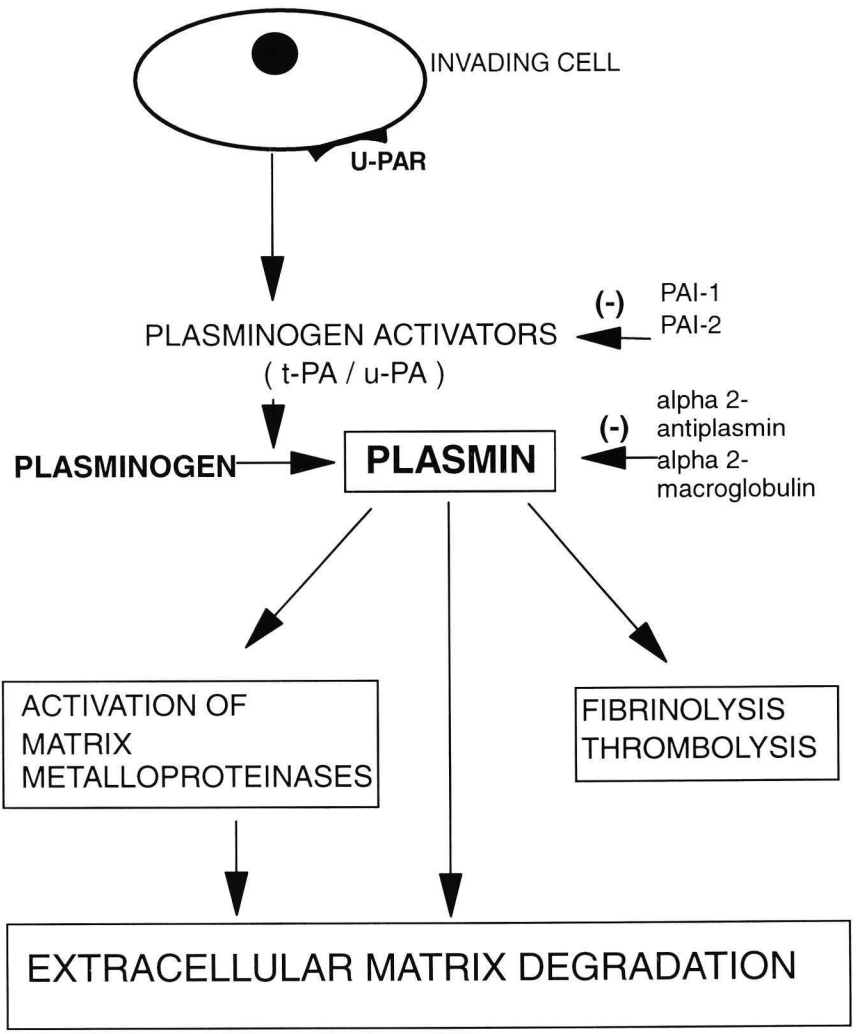

Figure 4. Simplified diagram of the plasminogen activation system. The pro-enzyme plasminogen can be converted to plasmin by u-PA and t-PA. Plasmin is involved in fibrinolysis, activation of matrix metalloproteinases and extracellular matrix degradation.

and genetically distinct PAs have been identified in mammals; tissue-type PA (t-PA) and urokinase-type PA (u-PA). Both enzymes are synthesized as single chain proteins.
Single chain u-PA is a pro-enzyme with 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 space and can be mediated by plasmin, kallikrein, factor XIIa or cathepsin (61-63).
Unlike u-PA, both single chain and two chain t-PA possess proteolytic activity.
Both PAs have a proteolytic domain, called the B chain, which has an active site containing histidine, aspartic acid and serine residues similar to that of other serine proteinases.
The A chain of both PAs has a growth factor domain homologous to the receptor-binding regions in epidermal growth factor, and a kringle structure like in plasminogen (53).
In t-PA, the function of the growth factor domain is unknown, whereas this domain in the amino-terminal region of u-PA contains an amino acid sequence that directs binding of u-PA to a specific, high affinity cell surface receptor (64).
An important structural difference between the two PAs is that t-PA has an additional kringle and a finger-like domain which are involved in binding of t-PA to fibrin. Activity of t-PA is strongly enhanced by binding to fibrin(ogen) (65). Because u-PA binds less well to fibrin and its activity is not stimulated by fibrin, it was previously thought that t-PA was mainly involved in fibrinolysis, whereas u-PA was more implicated in extracellular proteolysis (66,67). Recently, this sharp distinction has been abandoned, since many tumor cells have been found to produce t-PA besides u-PA (68) and u-PA has been found to play a role in dissolving fibrin (54)

inhibitors

Four serine proteinase inhibitors are of particular relevance to the PA/plasmin system.
The activity of plasminogen activators can be inhibited by specific inhibitors of which the plasminogen activator inhibitor-1 (PAI-1), PAI-2 and the plasmin inhibitor α_2-antiplasmin are most important (69).
PAI-1, believed to be physiologically most important (60,70), is abundantly present in the circulation. It is produced by a variety of cells, including endothelial cells, fibroblasts, smooth muscle cells and tumor cells (71). It regulates pericellular proteolysis by forming inactive complexes with both single and two chain t-PA, and two chain u-PA. PAI-1 is unstable and degenerates into a latent form unless its active conformation is stabilized by binding to the adhesive protein vitronectin. PAI-vitronectin complex is the predominant form of the inhibitor found in plasma. Vitronectin is also the site for attachment of PAI-1 to the extracellular matrix (70).
The distribution and occurence of PAI-2 are less well defined. PAI-2 is not normally found in plasma. It is synthesized in the placenta and by macrophages/monocytes. PAI-2 forms

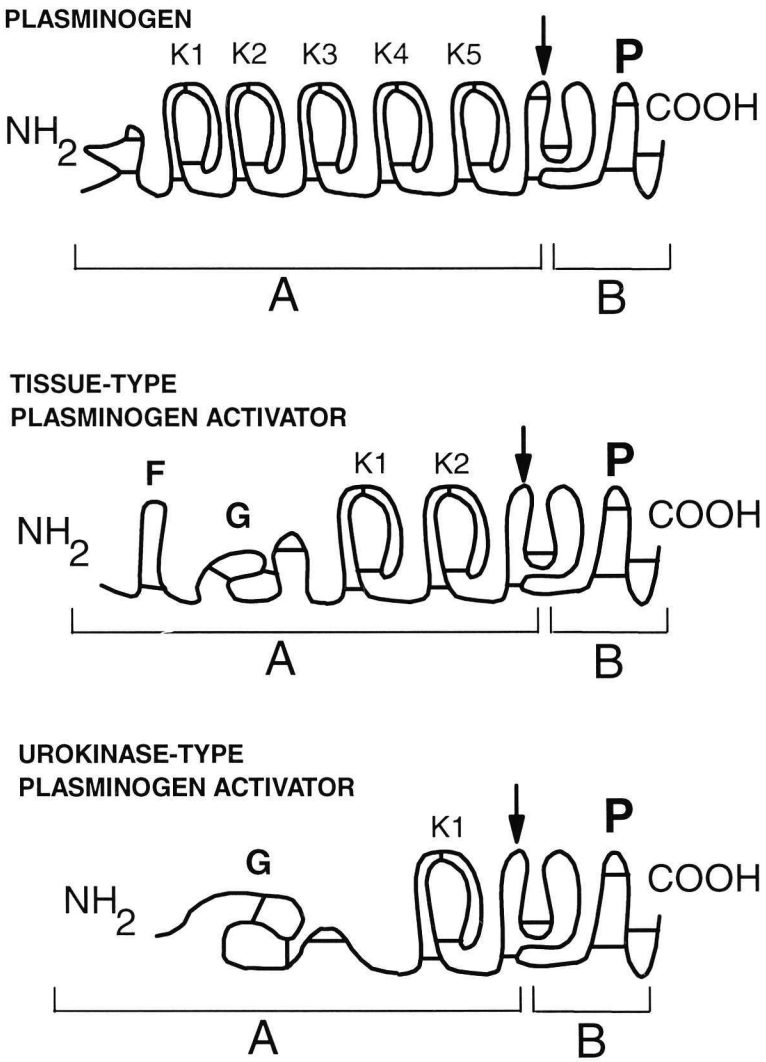

Figure 5. Schematic representation of plasminogen, t-PA and u-PA. K=kringle, P=proteolytic, F=finger and G=growth factor domain. Cleavage sites are indicated by arrows. A and B represent chains after cleavage.

inactive complexes with two chain and single chain t-PA and two chain u-PA, but the rate constants of inhibition are not as favorable as with PAI-1.
Protease-nexin 1 is a glycoprotein found in fibroblasts. This unspecific serine proteinase inhibitor reacts with u-PA, plasmin, trypsin and thrombin. The reactivity of protease-nexin 1 with PAs is less than that of PAI-1 and PAI-2. Its dual role in fibrin formation and removal is poorly understood. Inhibition of matrix dedradation by protease-nexin 1 has been reported (73).
α_2-Antiplasmin is the primary plasmin inhibitor in plasma. Occupation of lysine-binding sites by cell-surface proteins or specific extracellular matrix components, such as fibrin and fibronectin, protects plasmin from inhibition by α_2-antiplasmin (74).

Cell surface binding sites; plasmin and plasminogen activator receptors
Plasmin(ogen) and plasminogen activators can bind to cell surface receptors. Plasminogen is known to bind with different affinities to a wide variety of cells, including fibroblasts, peripheral blood leucocytes, lymphocytes and endothelial cells (58). Plasminogen-binding proteins have been characterized on the surface of rheumatoid synovial fibroblasts and are absent on normal synovial fibroblasts (75). The binding of plasminogen can be inhibited competitively by analogues of lysine, indicating that this interaction requires the lysine binding sites. Cell surface-bound plasmin is protected from its physiological inhibitors α_2-antiplasmin and α_2- macroglobulin (58,76). The cell surface, where (pro-)uPA and plasminogen are bound in close proximity, provides a suitable local environment for activation of plasminogen; activation of pro-uPA is markedly increased when it is receptor-bound and when plasminogen is simultaneously present. Furthermore, surface-bound plasmin, prevented from inhibition, accelerates activation of pro-u-PA (60) (figure 6).
(Pro-)uPA can bind to a specific cell surface receptor through its growth factor domain, located in the A chain (77). This receptor molecule has been demonstrated on many cell types, such as monocytes, granulocytes, endothelial cells, tumor cells and synovial fibroblasts (75,78). The amount of expression and the distribution of receptor varies between cell types and is subject to regulation by cytokines and growth factors (79,80).
The purified u-PA receptor (u-PAR) is a 55 kD protein consisting of three domains. The amino-terminal domain retains a (pro-)uPA binding site, whereas the presence of the other two domains is important in determining the high affinity of the u-PA/u-PAR interaction (81). U-PAR is inserted in the cell membrane by a glycosyl-phosphatidylinositol (GPI) anchor (81,82) and interacts with integrins, cellular adhesion molecules and vitronectin (83,84).
Receptor bound pro-uPA can be converted to its active form. In contrast to surface-bound plasmin, which escapes inhibition by its natural inhibitors, bound u-PA remains sensitive to PAI-1 and PAI-2 (85). The formed u-PA/PAI complexes are internalized by the LDL-receptor related protein/α_2-macroglobulin receptor (86) and degraded intracellularly. The receptor recycles back to the cell surface (79).

Binding of u-PA to its receptor has important functional consequences. The interaction increases the efficiency of plasminogen activation strongly (87). The expression and redistribution of u-PAR at the leading edge of migrating and invasively growing cells (88) creates a powerfull localized proteolytic system, that greatly enhances pericellular matrix degradation and cell invasion (89-91). This cell mediated potentiation of plasminogen activation can be abolished by blocking the u-PA binding site of u-PAR (92), as well as by low concentrations of lysine analogues which are antagonists of the cellular binding site of plasminogen (93,94)
In addition to plasminogen activation, u-PA also possesses signal transducing properties such as chemotactic activity and mitogenesis. The exact mechanisms need to be resolved. Binding of u-PA with vitronectin, and u-PAR and interaction with integrins, and growth factors seem to be involved (95,96)
Recent data suggest a physiological role of the cell-surface related u-PA/plasmin system in the regulation of MMP activity. Cellular binding of u-PA and plasminogen results in activation of the gelatinases MMP-2 and MMP-9, which may also associate with the cell surface. In the soluble phase, plasmin degrades gelatinase. Degradation and activation of MMPs occur in the presence of physiological concentrations of u-PA and plasminogen (97).
Table 2 summarizes the specifity of important components of the plasminogen activation pathway.

Table 2. Specifity of the components of the plasminogen activation system

Component	**Specificity**
Plasmin	laminin, fibrin, fibronectin, proteoglycans, gelatins, u-PA
t-PA	plasminogen
u-PA	plasminogen
PAI-1	t-PA, u-PA
PAI-2	u-PA, t-PA
protease-nexin 1	u-PA, t-PA, plasmin, trypsin, thrombin
u-PA receptor	pro-uPA, u-PA

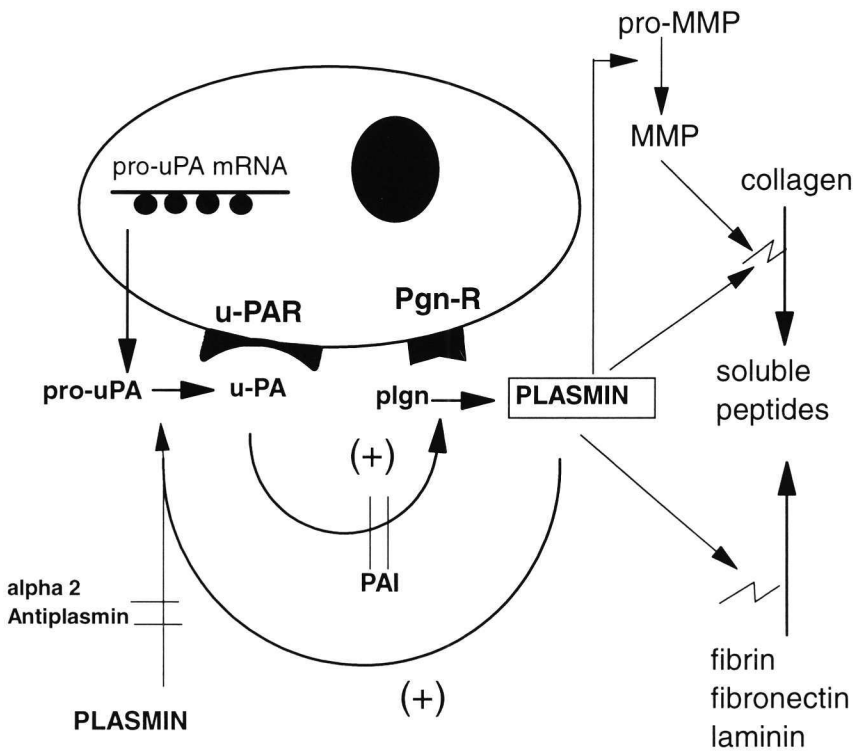

Figure 6. Diagram of the interaction between u-PA and plasminogen at the cell surface.

PLASMINOGEN ACTIVATION IN RHEUMATOID ARTHRITIS

The similarity of tumor infiltration to invasive growth of inflammatory tissue has led to the investigation of fibrinolytic enzymes and inhibitors in synovial tissue of patients with RA. Clinical and experimental evidence suggests a contribution of the PA system to joint destruction in RA, and almost all cells present in the joint have been shown to produce PAs and their inhibitors (PAIs) (98). In addition, increased levels of uPA and PAI in synovial fluid of RA patients, compared with corresponding plasma levels, indicate increased local production by the inflamed synovial tissue (99,100).
An immunohistochemical study, performed in 3 RA and 3 osteoarthritis (OA) patients, showed increased expression of u-PA and α_2 -plasmin inhibitor in RA synovial tissue (103). Chapter 2 of this thesis, describes the difference in expression of u-PA, t-PA, PAI-1, PAI-2 and u-PAR in synovial tissue of patients with RA compared OA. RA synovial fibroblasts not only show increased production of u-PA (102), but also, besides u-PAR, express novel proteins involved in plasminogen binding and activation (75). The enhanced expression of components of the PA system in rheumatoid synovial fluid has been associated with clinical severity of RA (103).
A pathophysiological role in bone and cartilage destruction has been suggested. Plasmin *per se* has proteolytic capacity, and can also activate other enzymes like stromelysin and collagenase (48,49,104). In an attempt to establish whether PA is necessary for bone resorption Leloup et al.(105) studied the effect of stimulation by parathyroid hormone (PTH) on osteoclast bone invasion. PTH enhanced u-PA production in mouse metatarsal bones, but the resorption process appeared to be independent of the presence of plasminogen or plasmin activity. On the other hand, osteogenic cells, such as osteoblasts and osteosarcoma cells have been shown to possess u-PA mediated, matrix degrading capacities (106,107). Increased expression of u-PA and u-PAR by activated osteoclasts (108) do leave the question of the pathophysiological role of PAs in bone resorption. One possibility is that PAs exert their action independent of their proteolytic activity. This is illustrated by the observation that the growth factor domain of t-PA appears to be involved in osteoclast formation (109).
PAs are believed to play a role in cartilage degradation but their involvement has still not been proven. The presence of plasminogen in chondrocyte cultures causes increased matrix degradation in the presence of IL-1 and TNF-α (110,111), implicating local production of PAs. Chondrocytes have been shown to be able to produce PAs and PAI-1 in increased amounts on stimulation with IL-1 (112). Cleavage sites for plasmin and u-PA have been found in the interglobular domain between the G1 and G2 globular domains of aggrecan core protein (113). The PA system could also be involved in cartilage degradation by its capacity to activate pro-MMPs. Procollagenase (MMP-1) can be partially activated by plasmin to produce a molecule which is further cleaved by stromelysin-1 (MMP-3). Soluble phase plasmin has been reported to be a very poor activator of 72-kDa (MMP-2)

and 92-kDa (MMP-9) gelatinases (114). Mazzieri et al. however, showed that binding of both plasminogen and u-PA to the cell surface resulted in activation of latent-MMP 2 and MMP- 9 (115). As cathepsin B, an activator of proMMP-3 also has been reported to activate pro-u-PA (116), a complex interactive cascade of cysteine proteinases, MMPs and PAs finally resulting in cartilage degradation can be envisaged.

On the assumption that PA is involved in bone and cartilage degradation, several studies have been performed in animal models of OA to assess a possible joint protective effect of anti-plasmin agents. Intra-articular administration of recombinant human protease nexin-1, an inhibitor of plasmin and u-PA has been shown to prevent Il-1β/fibroblast growth factor induced proteoglycan loss in rabbit knee (117). Tranexamic acid (TEA) is another anti-plasmin agent that has been studied in this respect. After oral administration it is rapidly distributed throughout extracellular compartments, including the synovial fluid (118). The molecular basis of its anti-plasmin activity is most likely due to its complementary structure, that blocks plasminogen-substrate and plasminogen-cellular binding sites (lysin binding sites). It has been demonstrated that intra-articularly administrated TEA prevented proteoglycan loss from cartilage of rabbits, subjected to medial meniscectomy (119). Vignon et al. (120) showed that TEA reduces cartilage destructive lesions and suppresses synovial membrane stromelysin and collagenase activity in osteoarthritic rabbits.

MATRIX METALLOPROTEINASES

In 1962, Gross and Lapière discovered an enzyme that could specifically cleave the collagen in the resorbing tail of the tadpole (121). Since the discovery of this collagenase, a whole family of enzymes has been described, that can degrade almost all proteins of the extracellular matrix. These enzymes, called matrix metalloproteinases (MMPs) because they require metal ions for their activity, are believed to play an essential role in the (patho-)physiological remodeling of tissue (122,123).
MMPs have a characteristic multi-domain structure and they can be divided according to their mode of zinc binding. All members of the family have a zinc ion and a calcium binding site in their catalytic domain, which mediates hydrolysis and autolytic cleavage. Each enzyme is secreted with a propeptide attached that has to be removed proteolytically to activate the enzyme.
The MMP superfamily includes 5 different groups of enzymes namely, collagenases (MMP-1, -8, -13), gelatinases (MMP-2 ,-9), stromelysins (MMP-3, -10, -11), membrane-type MMPs (MT-MMP-1, -2, -3, -4) and matrilysin (MMP-7) (122). Each group has its own substrate specificity (124).
MMPs are believed to fulfill an important role in the pathologic joint destruction in RA (48). Many cells, present within the inflamed joint, including synovial fibroblasts, chondrocytes, osteocytes, neutrophils, macrophages and endothelial cells, have been shown to produce MMPs, and increased concentrations of these proteinases have been found in RA synovial tissue and fluid (125-130).
MMPs are potent enzymes and they need to be carefully controlled. The main mechanisms involved in the regulation of MMP activity are; 1) Gene expression, synthesis and secretion. 2) Activation of pro-MMPs by removal of the pro-peptide. 3) Inhibition by specific MMP inhibitors.
A wide variety of cell types has the potential to degrade the extracellular matrix, but many of them secrete little or any MMP unless triggered. Numerous factors, both physical and chemical, are involved in the stimulation and suppression of MMP activity. A correlation between alteration of cell adhesion to the matrix, cytoskeletal architecture, and the expression of MMP genes has been described (130). Within the arthritic joint, MMP expresssion in fibroblasts, chondrocytes and macrophages is induced by pro-inflammatory mediators such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α) (131-133) and by several growth factors (134).
All MMPs are produced in a pro-enzyme form that requires activation before the enzyme is able to degrade its substrate. Activation of MMPs is effected by removal of a cysteine residue present in a N-terminal peptide, that previously blocked the active site zinc atom (122,135). Proteolytic cleavage of the propeptide of most MMPs can occur by a number of routes involving other extracellular proteinases, such as components of the plasminogen activation system as well as other MMPs or autolytic mechanisms (136) (Table 3).

Tissue Inhibitors of Metallo-Proteinases (TIMPs) are the major physiological regulators of MMP activity. They play an important role in controlling connective tissue breakdown by blocking the action of active MMPs and preventing activation of the proenzymes. Inhibition of MMPs occurs as a result of TIMP binding at the active site of MMPs (137).

Table 3. Proteolytic processing of propeptides of MMPs.

MMP	No	Exogenous activators	Activating
collagenase-1	1	kallikrein, chymase, plasmin, MMPs	MMP-2
collagenase-2	8	plasmin, MMP-3, MMP-10	
collagenase-3	13	MMP-2, MMP-3, MMP-10	MMP-2, MMP-9
stromelysins-1, -2	3, 10	plasmin, kallikrein, chymase, tryptase	MMP-1, MMP-9
		elastase, cathepsin G,	MMP-8, MMP-13
stromelysin-3	11	furin	
matrilysin	7	plasmin, MMP-3	MMP-2
metalloelastase	12		
gelatinase A	2	MMP-1, MMP-7, MMP-13	MMP-9, MMP-13
gelatinase B	9	plasmin, MMP-3, MMP-2	
MT1 MMP	14	furin	MMP-2, MMP13

GRANZYMES

Cartilage destruction in RA has mainly been attributed to serine proteinases and MMPs, produced by fibroblast-like synoviocytes, macrophages, chondrocytes, and polymorphonuclear cells (47,48). Recently, a set of serine proteinases, called granzymes (Gran), has been identified that may play a role in cartilage degradation (50,51). Granzymes, which include Gran A, a proteinase with trypsin-like activity and Gran B, a proteinase which specifically hydrolyses after aspartic acid residues, are soluble cytolytic proteinases able to induce apoptosis in target cells in the presence of perforin (138,139). After release of the lysosome-like granules by activated cytotoxic lymphocytes and natural killer cells, Grans may also exert extracellular effects (140,141). Gran 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 (142,143). Furthermore, these enzymes may be involved in remodeling of extracellular matrix, as illustrated by the capacity of Gran A to degrade basement membrane type IV collagen (144,145).
Several observations suggest a role of Grans in joint inflammation and destruction in patients with RA. In synovial fluid (SF) of patients with RA, lymphocytes have been shown to express Gran A messenger RNA (mRNA) (146-148). Recently, increased concentrations of soluble Gran B were found in SF of RA patients when compared with patients suffering from osteoarthritis or reactive arthritis (149). The levels of soluble Gran B were significantly higher in SF than in corresponding plasma samples indicating local production within the inflamed joint. These observations in SF are in line with those in rheumatoid synovial tissue (ST) where the presence of Gran A and Gran B (150-152) has been reported. The number of Gran 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 (151,152).

AIM AND CONTENTS OF THE STUDY

The mechanisms underlying cartilage and bone destruction in rheumatoid arthritis are poorly understood. Increased extracellular proteolysis involving metallo- and serine proteinases is thought to play a role in rheumatoid joint destruction (48). The collagenic structures of bone and cartilage can be degraded by MMPs secreted from neutrophils, synovial fibroblasts and macrophage-like cells. The role of MMPs in joint destruction seems beyond doubt. Among the serine proteinases, plasminogen activators and granzymes are of particular interest but their involvement in rheumatoid arthritis is less well established. In contrast to rheumatoid tissue remodeling, much evidence exists for involvement of the PA system in tumor associated extracellular matrix degradation. The aggressiveness of several solid tumors (reviewed by Emeis et al., 153) is strongly asssociated with their expression of u-PA and PAI-1, which both are independent prognostic determinants for relapse and survival. The similarity between tumor infiltration and invasive growth of transformed rheumatoid synoviocytes challenges to study the relationship between the PA system and rheumatoid joint destruction in more detail. A recently discovered set of serine proteinases, called granzymes, has been shown to be specifically increased in SF and ST of patients with RA. These proteinases are able to induce apoptosis in target cells. Little information exists about a possible role in extracellular matrix degradation.
The investigations presented here were designed to study:

The expression and distribution of several components of the plasminogen activation system in synovial tissue of patients suffering from erosive rheumatoid arthritis **(chapter 2).**

The capacity of the plasminogen activation to degrade bone and cartilage matrix *in vitro*, and the components involved **(chapter 3 and 4).**

The effects of intervention in the plasminogen activation system on matrix breakdown *in vivo* **(chapter 5).**

The capacity of granzyme B to degrade cartilage matrix and its expression at the pannus-cartilage interface in synovial tissue of patients with rheumatoid arthritis **(chapter 6).**

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CHAPTER 2

DIFFERENCE IN EXPRESSION OF THE PLASMINOGEN ACTIVATION SYSTEM IN SYNOVIAL TISSUE OF PATIENTS WITH RHEUMATOID ARTHRITIS AND OSTEOARTHRITIS

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SUMMARY

Proteolytic joint destruction in inflammatory and noninflammatory arthropathy is believed to be mediated, at least in part, by the plasminogen activation (PA) system. To further investigate possible involvement of the PA system we quantified immunoreactive urokinase-type plasminogen activator (u-PA), tissue-type plasminogen activator (t-PA), both plasminogen activator inhibitors (PAI-1 and PAI-2), and u-PA-receptor (u-PAR) in synovial tissue extracts of 14 patients with rheumatoid arthritis (RA) and 12 with osteoarthritis (OA). U-PA, PAI-1, PAI-2 and u-PAR concentrations were significantly higher in RA than in OA patients. T-PA antigen levels, were significantly lower in RA than in OA synovial tissue extracts. Immunohistochemistry was performed to compare the distribution and staining intensity of these components in samples of RA and OA synovial tissue. Intense immunostaining of u-PA, u-PAR, PAI-1 and, to a lesser degree, PAI-2 was observed predominantly in the synovial lining of RA patients. In OA patients, u-PA, PAI-1, PAI-2 and u-PAR were barely detectable. T-PA immunostaining was restricted to the endothelial side of vascular walls in both groups. We conclude that the observed increase of u-PA, u-PAR and PAI expression, distributed mainly in the synovial lining area of proliferative and invasively growing synovial tissue in RA patients, supports a pathogenic role for the PA system in destructive arthritis. Depressed t-PA mediated plasminogen activation might contribute to delayed intra-articular fibrin removal.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease, characterized by destruction of articular cartilage and bone. Proteolytic degradation of the extracellular matrix in inflammatory arthritis is considered to be mediated by proteinases like aspartic-, serine-, cysteine- and metalloproteinases (1). Hyperplastic inflamed synovial tissue overgrows and invades the articular cartilage and may be involved in the destruction of bone, tendons and ligaments by production of proteolytic enzymes (2-3).
Several studies suggest an important pathogenic role of the PA system in destructive joint disease (4-6), but detailed knowledge of the mechanism and components involved is lacking. The central enzyme, plasmin, is a broad spectrum serine protease, involved in fibrinolysis and thrombolysis as well as in degradation of extracellular matrix that is required for normal and pathological forms of cellular invasiveness (7). Plasmin is able to degrade extracellular matrix directly (8) and by activation of latent matrix metalloproteinases (9,10). It is produced as inactive plasminogen, that is converted into its active form by limited proteolysis of a single peptide bond by plasminogen activators. Two types of plasminogen activators (PA) have been characterized; tissue-type PA (t-PA), generally

viewed as being important in fibrin dissolution, and urokinase-type PA (u-PA), considered to be responsible for plasmin generation in processes involving tissue remodelling.
Other identified proteins of the PA system include: two plasminogen activator inhibitors, PAI-1 and PAI-2 (7) and a cell surface u-PA binding protein, the u-PA receptor (u-PAR). This receptor binds and localizes pro-u-PA as well as u-PA on the cell surface (11).
In patients with rheumatoid arthritis, a positive correlation between PA and PAI concentrations in synovial fluid and several markers of disease activity has been demonstrated (12-14). Increased levels of u-PA and PAI in synovial fluid of patients with inflammatory joint disease, compared with corresponding plasma levels are indicative of local plasminogen activation (15). Synovial fibroblasts, chondrocytes, endothelial-, mononuclear- and polymorphonuclear cells are capable of synthesizing u-PA, t-PA, PAI-1 and PAI-2. These cells could be responsible for the local production of PA and PAI in RA (16-19). It may be envisaged that local plasminogen activation, in the proliferative and invasively growing synovial membrane, promotes degradation of joint cartilage and bone.
In the present study, concentrations of several components of the PA system, i.e. u-PA, t-PA, PAI-1, PAI-2 and u-PAR, have been determined in extracts of synovial tissue of patients suffering from RA or OA, requiring surgery. Furthermore, the distribution of these components has been investigated by immunohistochemical staining of synovial tissue sections. In order to investigate a possible relationship between the level of synovial plasminogen activation and joint destruction a comparison is made between the findings in destructive inflammatory arthritis, e.g. RA, with those in non-inflammatory, degenerative joint disease, i.e. OA. The results of this study support the pathogenic importance of the PA system in destructive joint disease.

MATERIALS AND METHODS

Tissue sampling and extraction.
Specimens of synovial tissue were obtained from 14 RA patients and 12 OA patients who required joint surgery for severe disease. All RA patients, who fullfilled the established criteria (20), were operated on at the Orthopaedic Department of the University Hospital, Leiden. Patients with advanced OA, corresponding to grade 3-4 in the Kellgren classification system (21), were operated at the Department of Orthopaedics of Rijnland Hospital, Leiderdorp. Specimens of synovial tissue were immediately frozen in liquid nitrogen and stored at -80^{0}C until use.
For quantitative u-PA, t-PA, PAI-1, PAI-2 and u-PAR determination, tissue samples were homogenized in 1 ml 0.1% (v/v) Tween 80, 0.1 M Tris-HCl buffer (pH 7.5) per 60 mg wet tissue, as described previously (22). The homogenates were centrifuged twice at 8 x 10^3 *g* for 2.5 minutes, the supernatants collected and used in the assays. Protein concentrations were determined by the method of Lowry (23).

Quantitative Assays

u-PA antigen was measured with an enzyme-linked immunoassay that was developed in our laboratory and performed according to Koolwijk et al (24). The monoclonal antibodies used in this ELISA recognize all forms of u-PA (pro u-PA, active u-PA and the u-PA/PAI complex) with comparable efficiency. The detection limit is around 0.5 ng/ml. To assess u-PA activity, scu-PA and active tcu-PA were measured separately, using a biological immunoassay as described by Dooijewaard et al (25).

t-PA antigen was determined using the commercial ELISA Imulyse t-PA (Biopool, Umeå, Sweden). This method measures free t-PA antigen and t-PA/PAI-complexes with the same sensitivity. The detection limit is about 1.5 ng/ml.

PAI-1 antigen was measured with Innotest PAI-1 ELISA (Innogenetics, Antwerpen, Belgium), using monoclonal mouse-anti human-PAI-1 antibodies. This assay recognizes all forms of PAI-1 with the same sensitivity, with a detection limit of approximately 5 ng/ml.

PAI-2 antigen was measured with Tintelize PAI-2 ELISA (Biopool, Umeå, Sweden), using monoclonal mouse-antihuman-PAI-2 antibodies. This assay recognizes all forms of PAI-2 including the low molecular weight form and the glycosylated high molecular weight form of PAI-2. The detection limit of this assay is about 6 ng/ml.

u-PAR antigen was measured with Imubind u-PAR ELISA (American Diagnostica Inc, Greenwich CT), using polyclonal-rabbit antihuman-u-PAR. This assay recognizes soluble, native u-PAR as well as u-PAR/u-PA and u-PAR/u-PA/PAI-1 complexes. Detection limit is about 0.1 ng/ml. All enzyme-immunoassays were performed in duplicate.

Antibodies

Monoclonal antibodies against human u-PA (# 3698), polyclonal goat-anti human t-PA-antibodies (# 387), monoclonal anti human PAI-1 (# 380) and monoclonal anti human-u-PAR were purchased from American Diagnostica Inc. (Greenwich, CT). Goat polyclonal antibodies against human PAI-2 were a gift from E. Schüler (Behring Werke AG, Marburg, Germany). Characterization of these antibodies, including their positive and negative controls has been described in previous work (26,37).

Immunohistochemistry

Tissue samples of 5 RA and 5 OA patients were frozen in isopentane and stored at -80 ^{0}C. 4 μm Cryostat sections were air-dried overnight at room temperature and stored at -80 ^{0}C until use. Sections were fixed for 10 minutes in acetone at -20 ^{0}C before incubation with the primary antibody.

With monoclonal antibodies, a three-step avidin biotin peroxidase complex method was applied (Vectastain Elitekit, Vector Laboratories, Burlingame CA), as described by de Vries et al (26). Polyclonal antibodies were applied to the sections, washed and incubated with peroxidase-labeled rabbit anti-goat immunoglobulin. Bound antibodies were

visualized by using 3-amino-9-ethylcarbazole as a substrate for peroxidase as described (26).

Calculations and statistical analysis
Antigen concentrations were expressed as ng antigen per mg tissue protein. Differences between the median of the measured synovial tissue concentrations of u-PA, t-PA, PAI-1 and PAI-2 in the RA and the OA group were evaluated with the non-parametric Mann Whitney U test for unpaired parameters, utilizing the standard software package "Solo" (BMDP Statistical Software, Los Angeles, California, USA). Differences were considered significant when $p \leq 0.05$.

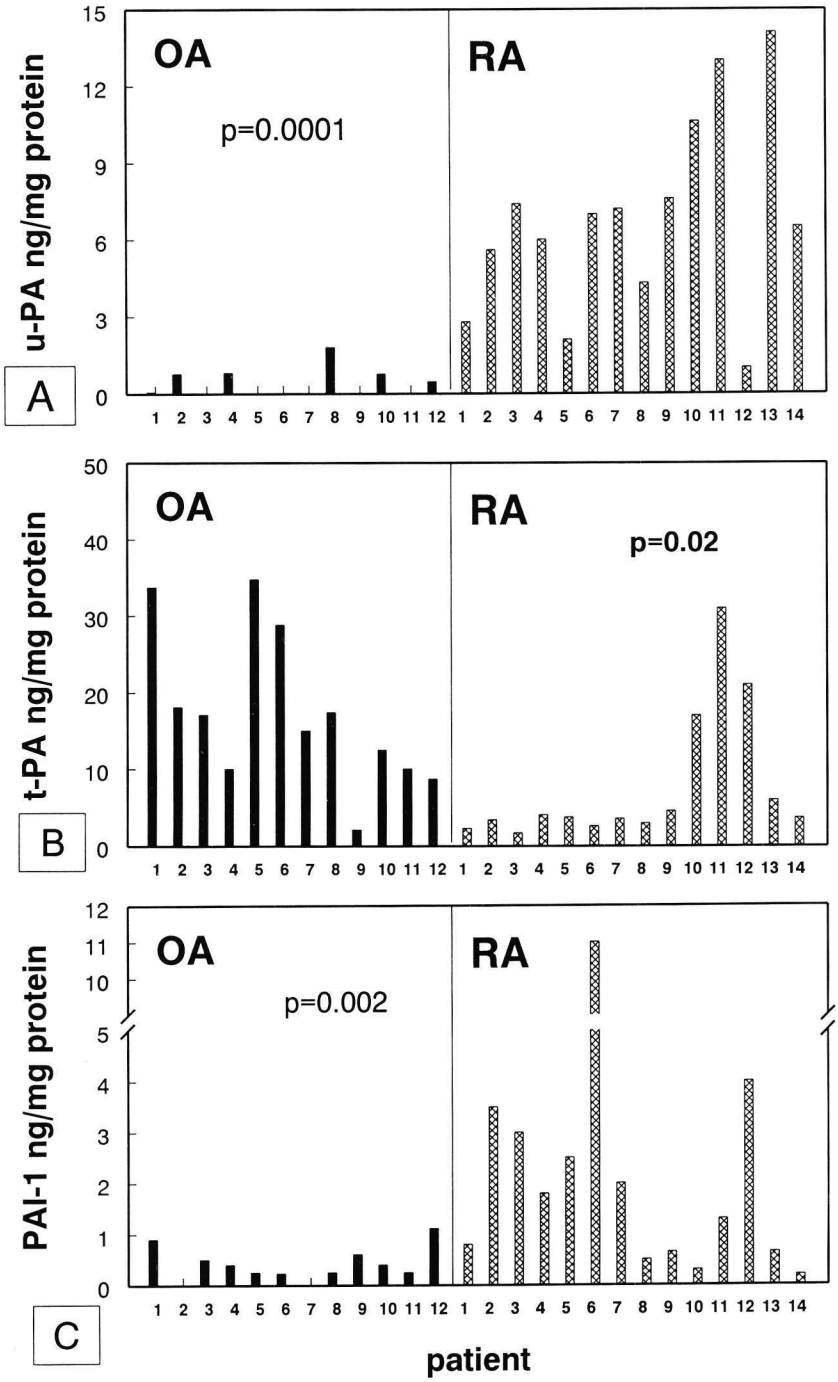
RESULTS

Quantitative assays
Enzyme-linked immunoassays were performed on homogenates of RA and OA synovial tissue samples to measure u-PA, t-PA, PAI-1, PAI-2 and u-PAR antigen levels.
The results in RA patients were compared with those in OA patients.
u-PA antigen could be detected in all RA patients and in 5 out of 12 OA patients.
When present, u-PA concentration was considerably higher in RA than in OA synovial tissue samples (figure 1A). Statistical analysis revealed a significantly higher median value in RA compared with the median value in OA (table 1). Sc-u-PA and active, tc-u-PA were determined with a bio-immunoassay. The levels of plasmin activatable sc-u-PA and active tc-u-PA in samples of synovial tissue of RA and OA patients were measured separately. Plasmin activatable scu-PA could not be detected in synovial tissue from either RA or OA patients, although active tcu-PA was found (data not shown).
T-PA levels were variable in both groups but could be detected in all available samples (figure 1B). In OA patient number 8, t-PA could not be measured because of shortage of synovial tissue. The median concentration of t-PA antigen in the RA group was significantly lower than in the OA group (table 1).
In all RA patients, and in 10 out of 12 OA patients, PAI-1 antigen was found in the synovial tissue (figure 1C). The median PAI-1 antigen level was significantly higher in the RA group than in the OA group (table 1).
PAI-2 related antigen was found in synovial tissue of 8 out of 14 RA patients and could hardly be detected in 1 out of 12 OA tissue samples. PAI-2 levels in RA synovial tissue were highly variable (figure 1D). The difference between both groups appeared to be statistically significant (table 1). In all samples of RA and OA patients, the presence of u-PAR antigen was detectable (figure 1E). Median u-PAR level was significantly higher in the RA than in the OA group (table 1).

Table 1.

Comparison of u-PA, t-PA, PAI-1, PAI-2 and u-PAR concentrations in synovial tissue of patients with rheumatoid arthritis (RA) and osteoarthritis (OA). Results are given as median and interquartile range (25%-75%). The median concentrations of u-PA, PAI-1, PAI-2 and u-PAR antigen in RA synovial tissue are significantly higher than in OA synovial tissue. The median t-PA antigen concentration is significantly lower in RA than in OA synovial tissue (Mann Whitney U test).

parameter (ng/mg protein)	RA (n = 14)	OA (n = 12)	p - value
u-PA	6.9(4.4-7.7)	0.03(0-0.77)	0.0001
t-PA	3.8(3.0-6.1)	17(10-28.8)	0.02
PAI-1	15.5(6.5-30)	3.3(2.4-5.5)	0.002
PAI-2	0.1(0-0.32)	0(0-0)	0.01
u-PAR	2(1.1-3.5)	0.8(0.6-1.4)	0.01


A
u-PA ng/mg protein
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RA
p=0.0001
B
t-PA ng/mg protein
OA
RA
p=0.02
C
PAI-1 ng/mg protein
OA
RA
p=0.002
patient

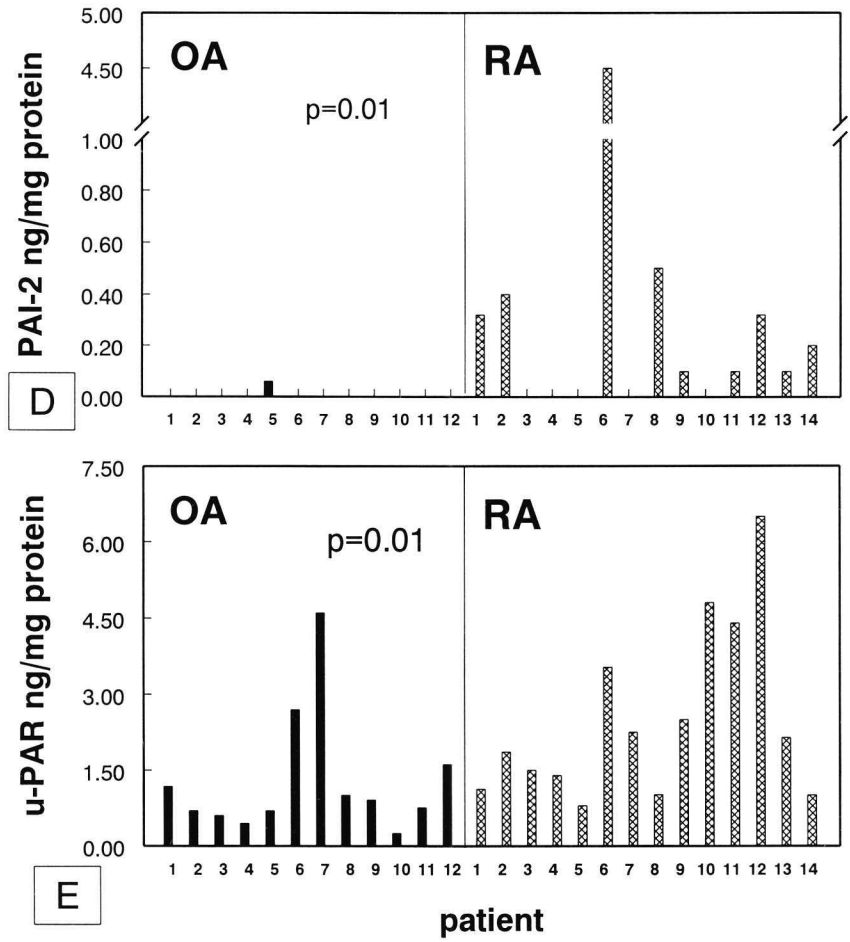


Figure 1. Antigen concentration in synovial tissue samples of 14 patients with rheumatoid arthritis and 12 patients with osteoarthritis. Antigen was measured in tissue extracts by enzyme immunoassay and expressed as ng/mg tissue protein. (A) u-PA; (B) t-PA; (C) PAI-1; (D) PAI-2; (E) u-PAR antigen. Statistical evaluation is summarized in Table 1.

Immunohistochemistry

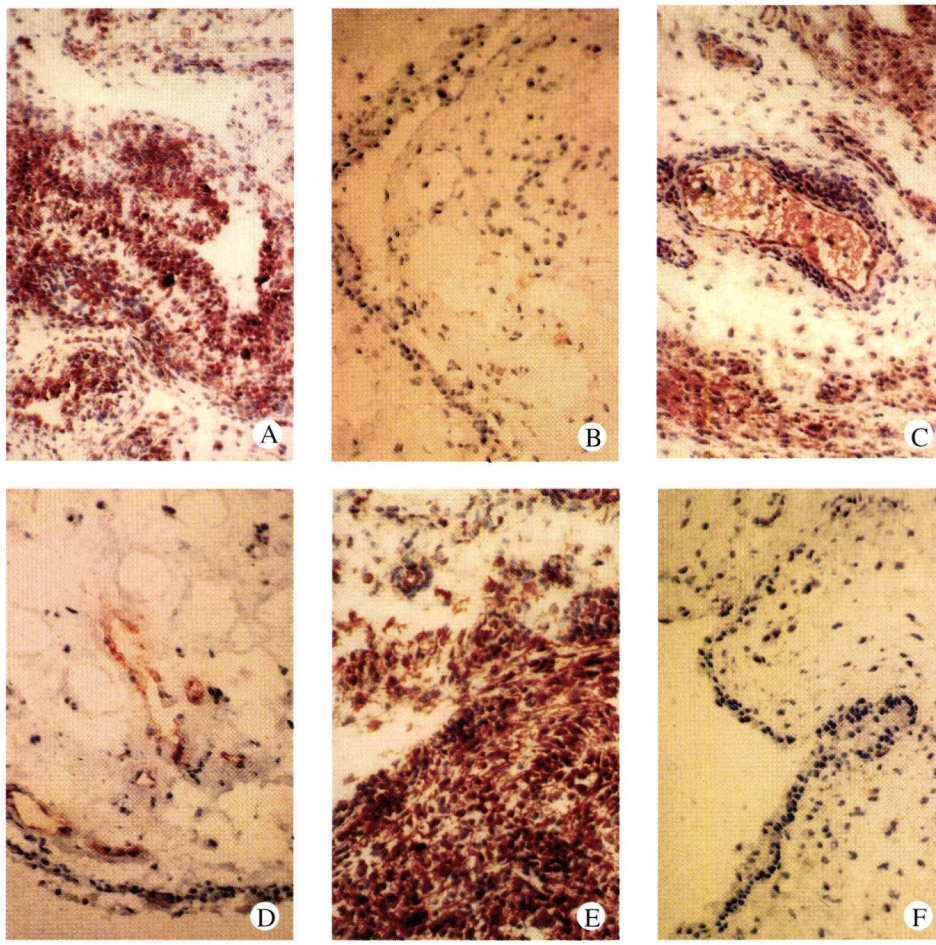
In order to investigate the localization of PA, PAI and u-PAR, immunohistochemistry was performed on sections of synovial tissue samples from 5 RA and 5 OA patients. Degree of expression of the various parameters is summarized in table 2, and shown in figure 2. Marked expression of u-PA was seen in all RA synovial tissues, especially in the synovial lining cell area and in giant cells (figure 2A), but also in some plasma cells in inflammatory cell infiltrates. Less intense immunostaining was observed in some bloodvessels, especially in the media of arterioles. In the OA synovial tissue samples hardly any u-PA could be detected (figure 2B), but when it was expressed, it was restricted to the synovial lining cell area. t-PA was observed in the endothelial cells of capillaries in both groups (figure 2C and D). Modest expression was seen in RA synovial lining area. No extravascular t-PA could be detected in OA synovial tissue. Strong PAI-1 immunostaining was seen in all RA synovial tissue samples. It was confined to the lining cell area (figure 2E) and capillaries. Hardly any PAI-1 was observed in OA synovial tissue samples (figure 2F). In only a few RA patients, PAI-2 was observed in parts of the synovial lining area (figure 2G). No PAI-2 could be detected in any sample of OA patients (figure 2H). Substantial u-PAR expression, predominantly in the lining cell area, was observed in synovial tissue of all RA patients (figure 2I). In OA synovial tissue no expression or only very weak expression of u-PAR was seen, mainly in association with mononuclear cells in the interstitium (figure 2J).

Table 2. Immunohistochemical expression of u-PA, t-PA, PAI-1, PAI-2 and u-PAR in synovial tissue of patients with rheumatoid arthritis (RA) and osteoarthritis (OA). ++ = strong expression, + = moderate expression, ± = weak expression, - = no expression.

	u-PA	t-PA	PAI-1	PAI-2	u-PAR
RA	++	+	++	±	++
OA	±	+	±	-	±

DISCUSSION

In the present study we investigated the expression and localization of several components of the PA system in samples of synovium, obtained from patients with RA and OA. As this enzyme system is believed to be involved in extracellular proteolysis leading to joint destruction, a comparison is made between these two patient groups, to further investigate a relationship between synovial tissue plasminogen activation and joint destruction.
RA synovial tissue homogenates were found to contain variable but significantly higher concentrations of u-PA, PAI-1, PAI-2 and u-PAR than OA synovium homogenates (figure 1 and 2, table 1). Immunohistochemically, the picture was more homogenous. u-PA, PAI and u-PAR appeared to be located mainly in the RA synovial lining. t-PA antigen levels in RA synovial tissue were lower than in OA synovial tissue. It was located predominantly in vessel walls and perivascularly.
The increase of u-PA, PAI-1, PAI-2 and u-PAR in tissue extracts of inflamed synovium presumably finds its origin in an enhanced local production. Increased levels of u-PA, PAI-1 and in some severe cases, PAI-2 in synovial fluid of inflamed joints compared with plasma (13-15) are indicative for generation within the joint. Furthermore cell- and tissue culture studies have demonstrated that synovial fibroblasts but also chondrocytes, monocytes/macrophages and endothelial cells are capable of synthesizing u-PA and PAI. *In vivo*, their production may be influenced by cytokines such as interleukin-1, tumor necrosis factor-α, and granulocyte macrophage colony stimulating factor (16-19). A local increase of u-PA at the expense of t-PA in RA synovial tissue compared with OA synovial tissue is in line with previous observations in synovial fluid (15) and in inflammatory bowel disease (27). The altered ratio of t-PA (high fibrin affinity) to u-PA (low fibrin affinity) could reflect a shift from fibrin(ogen) degradation towards extracellular matrix degradation. This might result in protraction of fibrin removal on the one hand and enhanced proteolytic degradation of joint bone and cartilage on the other hand (15)
The quantitative ELISA data and the immunohistochemical analyses show a clear difference between RA and OA synovial tissue. This obvious pattern points towards an increased expression of all those enzyme system components, believed to be involved in tissue remodelling. A comparison between neoplastic tissue and invasively growing inflamed synvovial tissue has been made before (7). Indeed, increased u-PA and PAI-1 levels in tissue extracts or sections, have been found in various malignant tumours (26,28-30) and support the involvement of the PA system in extracellular matrix degradation. But how could an increased u-PA production lead to higher proteolytic activity when its inhibitors PAI-1 and PAI-2 are elevated? The answer may be found in the upregulation of u-PAR expression at the cell surface. First, in vitro studies have shown that several cell types are capable of binding of u-PA at specific sites of the cell surface, whereas PAI was found at a different location (31).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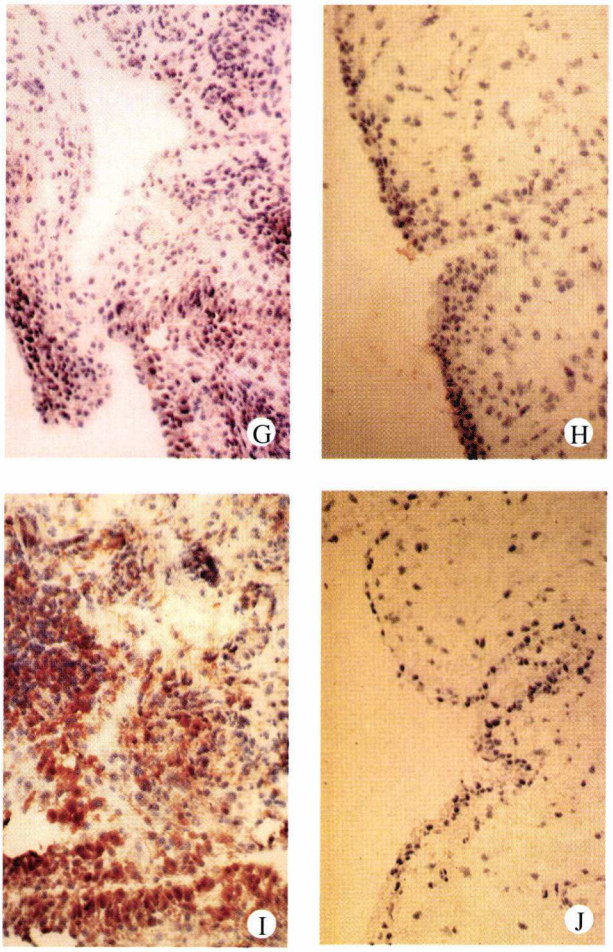

figure 2. Immunohistochemical staining for components of the plasminogen activation system in rheumatoid arthritis (RA) (A-E) and in osteoarthritis (OA) (F-J). Immunoreactivity for u-PA: (A) Distinct expression in synovial lining cells, and giant cells in RA; (B) Hardly any expression in OA; Immunoreactivity for t-PA: (C,D) Positive staining in endothelial cells in synovial blood vessels in RA and OA. Immunoreactivity for PAI-1; (E) Marked staining of synovial lining cells in RA; (F) No staining in OA. Immunoreactivity for PAI-2; (G) Focal expression in synovial lining cells in RA; (H) - No expression in OA. Immunoreactivity for u-PAR; (I) Distinct staining of synovial lining cells in RA, (J) No staining of synovial cells in OA. (Magnification 250x)

Second, a differential inhibition of soluble and cell surface receptor-bound u-PA has been demonstrated (32), allowing enzymatic activity of receptor-bound u-PA even in a PAI-rich environment. Third, colocalization of u-PA/u- PAR and plasminogen on the cell surface results in approximately 100-fold more efficient activation of plasminogen than in the fluid phase (33). Furthermore, plasmin bound to the cell surface is resistant to α2-antiplasmin (34). Fourth, the interaction of u-PA with its cell bound receptor has been shown to strongly enhance the degradation of extracellular matrix (35). Loss of the surface u-PA activity by blocking the interaction between the receptor and its ligand has been shown to inhibit invasive growth (36). These phenomenona could explain net local proteolytic activity in the presence of increased inhibitor. Our idea about the mechanism of plasmin mediated joint destruction is that increased u-PA production by inflamed hypertrophic and hyperplastic synovial tissue, overgrowing cartilage in a "tumor-like" manner, could lead to activation of the readily available plasminogen on the cell surface at sites occupied by u-PAR. This localized formation of active plasmin, capable of degrading extracellular matrix directly and by activation of matrix metalloproteinases, may subsequently result in directed, proteolytic degradation of bone and cartilage.
In conclusion the increased expression of u-PA, u-PAR, PAI-1 and PAI-2 in arthritic synovium compared with non-inflamed synovial tissue fits in the concept of a localized, u-PA mediated, plasmin dependent degradation of articular structures, finding its origin in inflamed synovial tissue.

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CHAPTER 3

BONE MATRIX DEGRADATION BY THE PLASMINOGEN ACTIVATION SYSTEM. POSSIBLE MECHANISM OF BONE DESTRUCTION IN ARTHRITIS

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SUMMARY

The observed increase of urokinase-type plasminogen activator (u-PA) and its receptor (u-PAR) in synovial tissue of patients with rheumatoid arthritis (RA) suggests pathophysiological involvement of the PA system in inflammatory joint disease.
In the present study we investigated the capacity of the PA system to degrade non-mineralized and mineralized bone-like matrix in vitro as a model for bone destruction. Transfected mouse LB6 cell lines, that expressed either human u-PA or u-PAR, were cultured separately and simultaneously on radiolabeled bone matrix in in the presence of plasminogen. Osteoblast-like murine calvarial MC3T3-E1 cells were used to produce a well characterized, highly organized bone-like matrix, that could be mineralized in the presence of β-glycerol phosphate. Bone matrix degradation was followed by the release of radioactivity in the culture medium.
U-PA producing cells, in contrast to u-PAR producing cells, degraded both non-mineralized and mineralized bone matrix. This effect could be inhibited by anti u-PA antibodies, as well as by tranexamic acid and by aprotinin, indicating that the degrading activity is u-PA mediated and plasmin dependent. Co-cultivation of a small portion of u-PA producing cells with u-PAR expressing cells resulted in a marked increase in degradation activity. Reduction of this potentiating effect by suramin or the aminoterminal fragment of u-PA, both competitive inhibitors of u-PA receptor binding, shows that this synergistic effect is due to binding of u-PA to u-PAR. U-PAR must be cell associated, as binding of u-PA to a soluble u-PAR prevented this enhancement.
The capability of the PA system to degrade bone matrix in vitro and the previously demonstrated increased expression of u-PA and u-PAR in synovial tissue of patients with RA further support a role of the PA system in the development of bone erosions.

INTRODUCTION

Destruction of the normal structure and function of the joint is a prominent feature of chronic inflammatory joint diseases. In rheumatoid arthritis (RA), extensive proliferation of synovial lining cells in the presence of infiltrating inflammatory cells leads to the formation of pannus that progresses over the articular cartilage and burrows into subchondral bone.
Three types of bone loss can be distinguished in RA: Generalized and periarticular osteoporosis, mediated by factors influencing the cycle of bone formation and resorption, and cortical bone defects at the joint margin site. These bony erosions may be due to the action of excessive amounts of proteolytic enzymes at the invading edge of hyperplastic synovial tissue (1). Several classes of proteolytic enzymes are held responsible for this extracellular matrix degradation and they may all be involved directly or by

augmenting the effectiveness of one another (2). The plasminogen activation (PA) system has been studied intensively in relation to tissue remodelling and cell migration. The central enzyme, plasmin, is a broad spectrum serine protease, involved in fibrinolysis and thrombolysis as well as in extracellular matrix degradation, required for normal and pathological forms of cell migration and tissue remodelling (3). It is able to degrade extracellular matrix directly (4), as well as by activating latent matrix metalloproteinases (5). Plasmin is produced as inactive plasminogen, that is converted to its active form by plasminogen activators like urokinase-type plasminogen activator (u-PA) and tissue type plasminogen activator (t-PA) (6). A specific cellular receptor for u-PA (u-PAR) (7) binds u-PA and localizes it at specific sites on the cell surface, e.g. cell to cell and cell to matrix contact sites (8).
A pathophysiological role of the PA system in destructive joint disease has been suggested before (9-11). Positive correlation between both u-PA and plasminogen activator inhibitor (PAI) concentrations in rheumatoid synovial fluid and markers of disease activity (12,13) and high expression of u-PA, u-PAR and PAI in synovial tissue of patients with RA (14) has been demonstrated. Although these observations suggest involvement of the PA system in destructive arthropathy, detailed knowledge of the mechanisms and components involved is lacking. It is unknown whether local expression of PA components by synoviocytes, exhibiting a "tumor-like expansion", contribute to the development of bony erosions. To further investigate a possible role of the PA system in this type of bone destruction, we studied the potential of this enzym system to degrade bone matrix *in vitro*. The model we used is based on an in vitro complementation system (15). Mouse fibrosarcoma cell line cells transfected with and expressing either human u-PA or human u-PAR, were cultured on a ^{3}H-labeled extracellular bone matrix. The role of u-PA and the effect of u-PA/u-PAR interaction in degradation of both non-mineralized and mineralized bone matrix is investigated.

MATERIALS AND METHODS

Cell culture
Mouse LB6 cells are fibroblast-like cells. This cell-line has been proven to be a suitable host to express different components of the human PA system (15). We used cells, kindly provided by Prof. F. Blasi, transfected with the cDNA for either human pro-u-PA (Clone-F) or human u-PAR (Clone-19) as well as untransfected cells (LB6).
The production of human pro-u-PA or u-PAR by transfected cells has been described previously (16,17). It has been shown that neither mouse u-PA, nor interstitial collagenases are produced by these cells. Furthermore, LB6 cells can be considered to lack binding capacity for human u-PA because binding of u-PA to its receptor is largely species specific (7,18).

The Amino terminal fragment of u-PA (ATF), which contains the receptor binding domain of u-PA but lacks the protease domain, has been produced by LB6-ΔF cells, kindly provided by Prof. F. Blasi, which were transfected with the ATF-u-PA cDNA as described (19). Conditioned medium, containing ATF in a concentration of approximately 2 μg/ml, was harvested from confluent cultures of LB6-ΔF cells. ATF concentration was determined by u-PA ELISA as described by Binnema et al. (20), after correction for the difference in molecular weight of ATF and u-PA.
A truncated form of the human u-PAR, lacking the carboxyterminal attachment site for the GPI-anchor, soluble u-PAR (Sol R), has been prepared from transfected CHO-cells, kindly provided by Dr. U Weidle, as has been described (21). Conditioned medium contained Sol R in a concentration of approximately 2 μg/ml, measured by u-PAR ELISA (American Diagnostica Inc. Greenwich, CT).
All cells were cultured in Dulbecco's Modified Eagle's Medium, 2 mM glutamic acid (GIBCO Life Technologies, Grand Island, NY 14022, USA) supplemented with 10% (v/v) FCS, 100 IU/ml penicillin G, 100 μg/ml streptomycin and 0.5 μg/ml amphotericin B (Boehringer Mannheim, Mannheim, Germany), at 37 ^{0}C in air with 5% carbon dioxide.

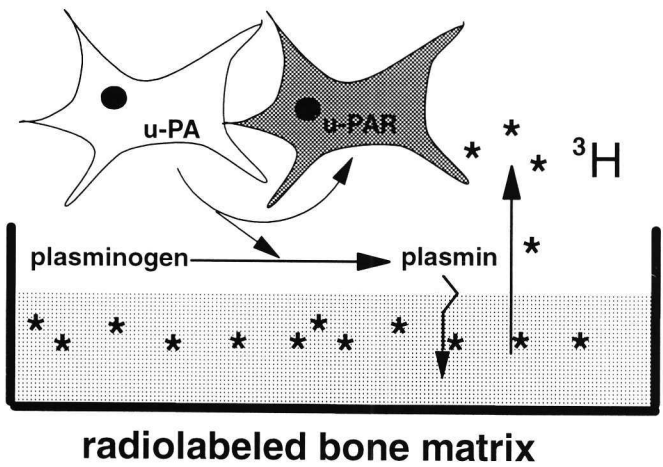

Figure 1. A schematic representation of mouse fibroblasts, expressing either u-PA or u-PAR, that were cultured on a radiolabeled bone matrix in the presence of plasminogen.

Formation of ^{3}H-labeled-, non-mineralized- and mineralized bone matrix.
The MC3T3-E1 mouse calvaria-derived cell line is a well characterized osteoblast culture system in which MC3T3-E1 cells display osteoblast-like characteristics, providing a suitable model of osteogenesis analogous to in vivo bone formation (22). When cultured in ascorbic acid containing medium, these cells have been shown to express a differentiated osteoblast phenotype including induction of alkaline phosphatase, osteocalcin, ostepontin, synthesis of type I collagen and formation of an extracellular collagenous matrix (23-25). Mineralization of extracellular matrix can be stimulated by β-glycerol phosphate but it only takes place when the cells have fully developed their osteoblast characteristics (26). Ultrastructural observations by Franceschi et al. (27) have indicated that the extracellular bone-like matrix is highly organized and contains well banded collagen fibrils.
We produced extracellular bone-like matrix by growing stock cultures of MC3T3-E1 cells in 162 cm^2 culture flasks containing "total growth medium": DMEM Glutamax supplemented with 10 % (v/v) foetal calf serum (FCS), 50 µg/ml ascorbic acid, 100 IU/ml penicillin G, 100 µg/ml streptomycin, and 0.5 µg/ml amphotericin B.
The cultures were incubated at 37 ^{0}C, in an environment of 95% air and 5% carbon dioxide. Culture medium was changed on the first day after seeding and then every 72 h. After 7-9 days in culture but before they reached confluence, cells were passed using 0.12% (w/v) trypsin (for 15 minutes) to achieve cell detachment. Their number was determined by direct counting with a hemocytometer. Cells were plated evenly into 24 well plates, coated with 0.5% gelatine (w/v) at an initial density of 1*10^4 cells/well. After 4-5 days (11-14 days after the initial seeding), when confluence in a lamellar pattern and partial multilayering of the cells occurred, media were removed and replaced by medium containing 1 µCi/ml ^{3}H Amino Acid mixture (Amersham Life Sciences, Amersham, Buckinghamshire, United Kingdom) to create a non-mineralized radiolabeled extracellular bone matrix. In order to create a mineralized bone matrix, "total growth medium" was supplemented with 10 mM β-glycerol phosphate and 15 mM HEPES buffer, as described previously (28). Extracellular matrix mineralized from day 7 onwards (approximately 13 days after initial seeding). Radiolabeled medium was changed every 72 h. Until day 14 (approximately 25 days after initial seeding), no mineralization of matrix was observed when no β-glycerol phosphate was added to culture medium.
At this time point, cells were lysed with 0.5 ml/well 0.5% (v/v) Triton X-100 in PBS. Cytoskeleton was removed by 0.5 ml/well 25 mM ammoniumhydroxide treatment, and proteinase activity was blocked by adding 0.5 ml 1 mM phenyl methane sulfonyl - fluoride (PMSF). Unincorporated ^{3}H-amino acids were washed from the remaining matrix using two water washes and 75% (v/v) ethanol. Matrices were dried and stored at -20 ^{0}C until use.

Bone matrix degradation

Before usage for degradation experiments, the ^{3}H-labeled bone matrices, described above, were slowly thawed at room temperature in DMEM. To assay for matrix degradation, cells ($1*10^{5}$/well) were seeded onto the matrix in culture medium and in medium supplemented with specific inhibitors (figure 1). All experiments were performed in FCS-containing medium to keep cells viable during the relatively long incubation period. Since this FCS contains various amounts of plasminogen, we added 0.14 μM purified plasminogen to culture medium to prevent influence of variations in plasminogen content. Degradation of bone matrix was assessed in a monoculture of control-LB6 cells, pro-u-PA-, or u-PAR producing cells as well as in cocultures, containing 5%, 7.5%, and 10% pro-u-PA producing cells respectively, in combination with either u-PAR producing cells or control LB6 cells. In order to be able to observe both stimulating and inhibiting effects, we chose a maximal degradation of around 60%. Such levels were reached after 72 hours of incubation.

The following inhibitors were added to different wells; polyclonal anti-human u-PA IgG (20), 30 μg/ml, polyclonal anti-human t-PA (29), 30 μg/ml, Aprotinin (Trasylol), a potent inhibitor of plasmin at a final concentration of 100 U/ml, tranexamic acid, an inhibitor of plasminogen activation, at a concentration of 1 mM, conditioned medium, 100 μl/ml, containing either ATF (final concentration approximately 0.2 μg/ml), or a soluble form of u-PAR (final concentration approximately 0.2 μg/ml), or suramin, a nonspecific inhibitor of receptor-ligand interaction, 60 μg/ml (30).

After 72 h of incubation the supernatant medium was removed. The degradation of the ^{3}H-labeled matrix was determined by assaying the amount of radioactivity, released in the medium, by liquid scintillation counting. The remaining matrix was degraded with 0.25% (w/v) trypsin, 0.1% (w/v) collagenase in PBS (1 h at 37 ^{0}C) and the amount of radioactivity in the matrix assessed. Matrix degradation was expressed as percentage ^{3}H, released in the medium of the total amount of ^{3}H, released by the cells during the 72 hours incubation period, plus that solubilized from the remaining matrix by trypsin/collagenase treatment.

Calculations and statistical analysis

All measurements were performed in triplicate. The results were given as the mean and standard error of the mean. Differences between degradation within one cell type were calculated with the Student's t-test for paired samples. Degradation levels brought about by different cell types were compared with Student's t-test for independent samples. Difference in degradation caused by co-culture of various percentages of u-PA producing cells with either u-PAR producing cells or untransfected LB6 cells was calculated by multiple regression. All calculations were performed utilizing the standard software package SPSS for MS Windows.

RESULTS

Bone matrix degradation by monocultures of u-PA producing cells, u-PAR producing cells and untransfected LB6 cells.

Degradation of the ^{3}H-labeled, mineralized and non-mineralized bone matrices by three individual cell lines, expressing either human u-PA, u-PAR or none of these components, was studied separately. The degree of matrix degradation was calculated from the release of radioactivity in the medium and expressed as percentage of the total radioactivity. As shown in figure 2, only u-PA producing cells were able to degrade both the non-mineralized and mineralized bone matrix. Cells transfected with the gene coding for human u-PAR and non-transfected LB6 cells, achieved negligible levels of degradation. Anticatalytic antibodies against human u-PA, the inhibitor of plasminogen activation tranexamic acid and the plasmin inhibitor aprotinin, strongly inhibited the degradation of both the non-mineralized and mineralized bone matrices. Addition of anti-human t-PA antibodies had no effect on the degradation (figure 2A and 2B). These results demonstrate the capacity of u-PA mediated plasminogen activation to degrade, both non-mineralized- and mineralized bone matrix.

Bone matrix degradation by a co-culture of u-PA producing cells and either u-PAR producing cells or LB6 cells.

The effect of the interaction between u-PA and cell surface u-PAR on bone matrix degradation was studied in co-culture experiments. u-PA producing cells were co-cultured with either u-PAR producing or untransfected LB6 cells in various ratios (0%-10%) on both non-mineralized and mineralized bone matrix. The effect of interaction is expected to occur most clearly at low percentages of u-PA producing cells, when the conversion of pro-u-PA to u-PA and thus the u-PA concentration is the rate limiting factor. The results of these degradation experiments are shown in figure 3. On non-mineralized bone matrix degradation was observed in both u-PA/LB6 and u-PA/u-PAR co-cultures. However, in the presence of the receptor, lower percentages of u-PA producing cells were able to bring about degradation. Furthermore, the degradation levels were higher in u-PA/u-PAR co-cultures than in u-PA/LB6 co-cultures at comparable percentages of u-PA producing cells. Apparently, the presence of u-PAR stimulates the degradation of this non-mineralized bone matrix by low percentages of u-PA producing cells (figure 3A). Mineralized bone matrix degradation by low percentages of u-PA producing cells was only observed in the presence of u-PAR, the level of degradation being dependent upon the percentage of u-PA producing cells.

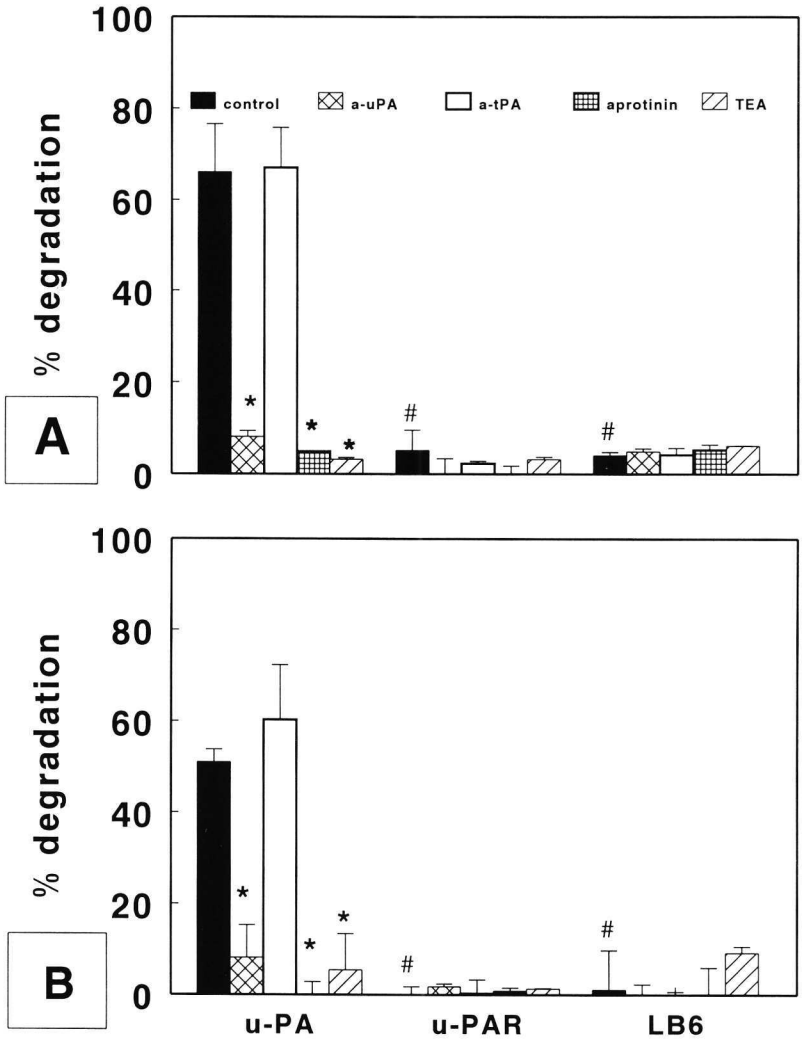

*Figure 2. In vitro degradation of non-mineralized (panel A) and mineralized (panel B) ^{3}H-labeled bone-matrix by u-PA producing cells (u-PA), u-PAR producing cells (u-PAR) and untransfected LB6 cells (LB6) in the presence of plasminogen. The effect of the addition of anti-u-PA anti-bodies (30 μg/ml), anti-t-PA antibodies (30 μg/ml), aprotinin (100 U/ml) and tranexamic acid (1 mM) to the culture medium on matrix degradation is shown. Results are given as the mean and standard error of the mean radioactivity, released in the medium as a percentage of the total radioactivity. (n=3). *Statistically significant different compared with u-PA control cells ($p < 0.01$; t-test for paired samples). #Statistically significant different compared with u-PA control cells ($p < 0.01$; t-test for unpaired samples).*

However, u-PA/LB6 co-culture, in a ratio up to 10% u-PA producing cells, hardly achieved any destruction of the mineralized bone matrix (figure 3B). These experiments show that the presence of u-PAR potentiates the capability of u-PA mediated plasminogen activation and the subsequent break-down of bone matrix. This stimulating effect of u-PAR was most clearly seen in mineralized bone matrix degradation.
To further investigate the functional role of u-PA/u-PAR interaction the following inhibition experiments were performed. First conditioned medium, containing the receptor-binding, non-proteolytic, aminoterminal fragment (ATF) of u-PA was added to a culture of 7.5% u-PA producing cells with 92.5% of either u-PAR producing cells or control LB6 cells in order to competitively inhibit the binding of u-PA to its receptor. Furthermore, the role of cell bound u-PAR was investigated by supplementation of a soluble form of this receptor (Sol R) which competes with the cell bound u-PAR for binding with u-PA. Finally, suramin, a potent, nonspecific inhibitor of receptor-ligand interactions was added to both types of matrices. It could be shown that interference with u-PA/cell bound u-PAR interaction by addition of aminoterminal fragment, soluble receptor or suramin, inhibits degradation by the u-PA/u-PAR co-culture of both non-mineralized bone matrix (figure 4A) and mineralized bone matrix (figure 4B).
In u-PA/LB6 co-culture, no significant effect was observed after addition of these competitive inhibitors, as expected. These experiments indicate that receptor binding is necessary for stimulation of u-PA mediated bone matrix degradation. Furthermore, receptor binding per se seems to be not sufficient, for u-PA must be attached to the cell surface in order to achieve enhancement of bone matrix degradation.

DISCUSSION

To investigate a possible pathogenic role of the PA system in the development of bone erosions in RA, we assessed the capacity of the PA system to degrade bone-like matrix *in vitro*.
PA-mediated degradation has been observed in various extracellular matrices, e.g. matrices produced by smooth muscle cells or tumor cells (4,31,32). In general the matrices used in these experiments contain either type IV collagen (33) or a combination of type I, III and IV collagen (34) and structurally they are not highly organized.
The extracellular matrix in our experiments was produced by MC3T3-E1 mouse calvaria-derived cells, a cell-line that has been reported to display osteoblast-like characteristics, providing a suitable model of osteogenesis analogous to in vivo bone formation (26). During the initial phase, these cells produce type I collagen which is deposited in an immature extracellular submatrix.

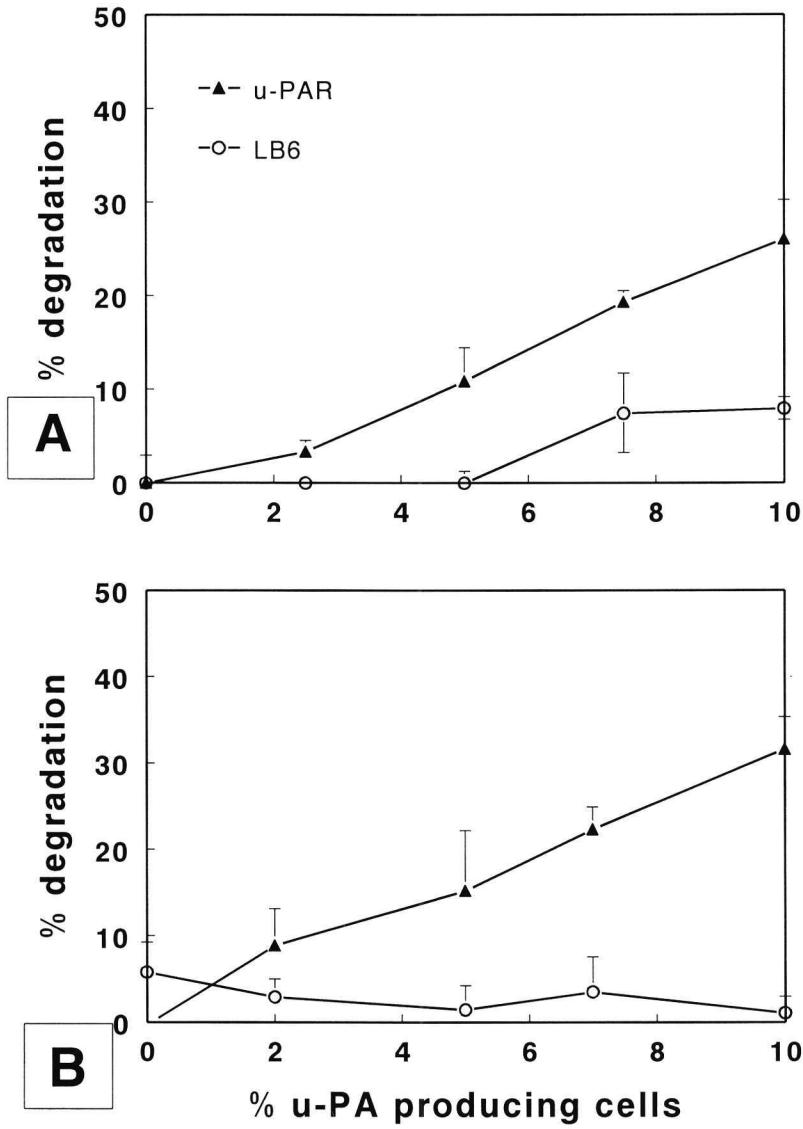

Figure 3. Degradation of non-mineralized (panel A) and mineralized (panel B) bone matrix by various low percentages (0-10%) u-PA producing cells (u-PA) cultured with either untransfected LB6 cells (LB6) or u-PAR producing cells (u-PAR). Measurements were performed in triplicate. Results are given as the mean and standard error of the mean radioactivity, released in the medium as a percentage of the total radioactivity. Difference in degradation between u-PA/LB6 and u-PA/u-PAR is statistically significant for both matrices ($p < 0.001$;multiple regression).

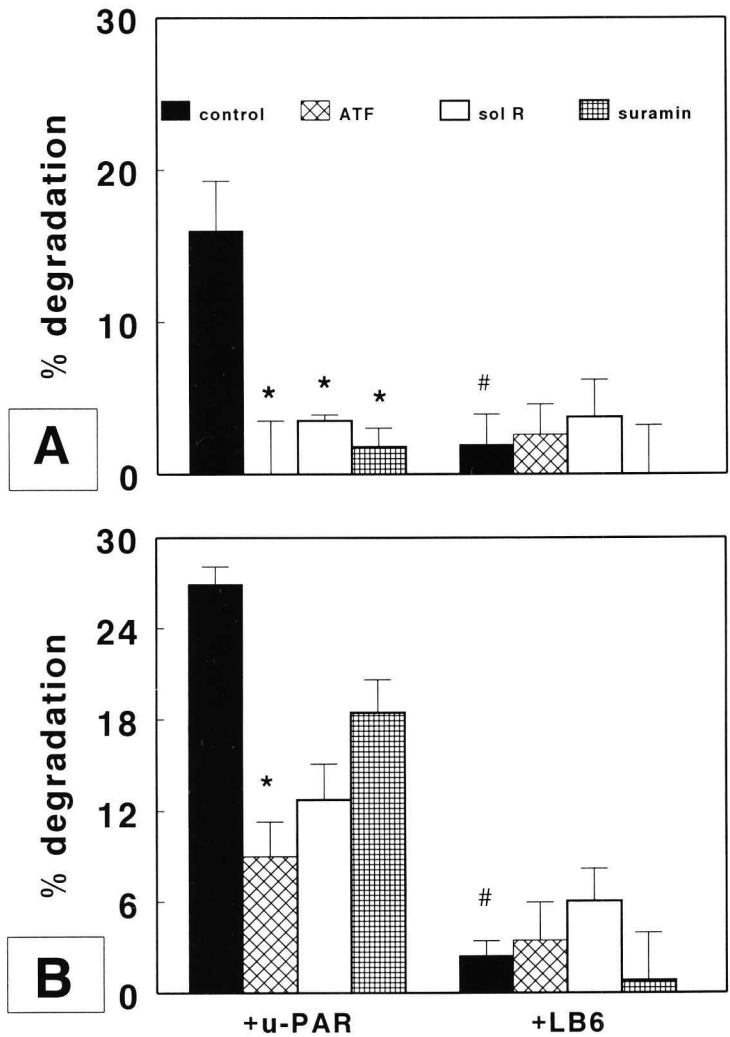

*Figure 4. Degradation of non-mineralized and mineralized bone matrix by co-cultures of u-PA producing cells (u-PA) with either LB6 control cells (LB6) or u-PAR producing cells (u-PAR) after supplementation with 100 µl/ml conditioned medium containing either the receptor-binding u-PA antagonist; ATF-u-PA (ATF, final concentration 0.2 µ/ml), or the soluble-, cell-surface detached u-PAR (Sol R, final concentration 0.2 µ/ml). Finally 60 µg/ml suramin (Sur) is added. Results are given as the mean and standard error of the mean radioactivity, released in the medium as a percentage of the total radioactivity (n=3). *Statistically significant different compared with +u-PAR control cells ($p < 0.02$; t-test for paired samples). #Statistically significant different compared with u-PA control cells ($p < 0.01$; t-test for unpaired samples).*

Bone specific characteristics of these cells are reflected by expression of alkaline phosphatase, osteocalcin, osteopontin, bone sialoprotein but also of receptors for 1,25-di(OH)vitamin D_3 (35), PTH (36), steroid hormones (37) and the production of growth regulating factors (38). As the extracellular matrix becomes mature, it shows a bone-characteristic organized collagen banding pattern. The bone-nature is evident from the appearance of mineralization, resulting inthe production of a solid sheet of mineralized matrix.

Our results show that untransfected LB6 cells, that lack human u-PA and its receptor were not able to bring about significant bone matrix degradation above background level. Matrix degradation, however, could be accomplished by using LB6 cells, that were transfected with the human gene for u-PA. Inhibition of this degradation by anti u-PA antibodies, tranexamic acid and aprotinin indicates the functional involvement of u-PA and plasminogen activation.

Enhancement of both non-mineralized and mineralized bone matrix degradation was seen when a limited number of u-PA producing LB6 cells were co-cultured with u-PAR producing LB6 cells, the latter having no or very low capacity to degrade the bone-like matrix. The enhancing effect of the u-PA/u-PAR interaction was more pronounced in the mineralized than in the non-mineralized bone matrix. This indicates that the presence of u-PAR is a prerequisite for degradation of mineralized bone matrix, when limited amounts of u-PA are available. Reversal of the enhancement by addition of suramin, or ATF, both competitive inhibitors of u-PA/u-PAR binding, shows that this potentiating effect is most likely due to binding of u-PA to its receptor. Our observation that addition of soluble, non-surface associated u-PAR, decreases the efficiency of degradation indicates that binding of u-PA to its cell surface receptor is required for efficient proteolytic degradation of bone-like matrix. This efficiency might be attributed to increased rate of plasminogen activation when u-PA and plasminogen are bound in close proximity to the cell surface (39,40,41,49). Furthermore, cell membrane bound components of the PA system probably may be less susceptible to proteinase inhibitors being present in FCS.

In vivo, local overexpression and binding of PA components by invasively growing synoviocytes (10,42) or recruited osteoclasts may contribute to erosion of underlying bone. U-PA, especially when bound to its receptor could provide proteolytic activity required for extracellular matrix degradation, including the activation of latent matrix metalloproteinases. In this concept synoviocytes exhibit "tumor-like" expansion and invasive behaviour like cancer cells. Indeed, the invasive and metastatic capacity of tumor cells can be inhibited by saturation or u-PAR with inactive u-PA or u-PA antagonists *in vitro* and *in vivo* (43).

Several observations provide evidence for the involvement of the PA system in bone resorption. Osteoblasts and osteoclasts, stimulated by bone-resorbing hormones or

lowered pH, have been shown to be able to produce plasminogen activator (44-46) and u-PAR (47). Thomson et al. (45) demonstrated the involvement of a plasminogen-plasmin-metalloproteinase cascade in type I collagen degradation by mouse osteoblasts. In an attempt to further establish whether PA is necessary for bone resorption Leloup et al. (46) studied the effect of stimulation by parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D_3 on osteoclast bone invasion and mineral resorption. PTH enhanced u-PA production in mouse metatarsal bones but the resorption process was independent of the presence of plasminogen or the activity of plasmin. This apparently contrasts with plasminogen dependence that has been reported for the degradation of collagen and bone matrix in vitro (45,48). One explanation could be that *in vivo*, plasmin contributes to degradation in a nonlimiting manner with alternative pathways available. These plasminogen-independent pathways, which may be efficient in whole-bone explants, could be missing or not active in cultures of isolated cells.
In conclusion, our findings demonstrate the capability of the PA system to degrade bone-like matrix in vitro and provide evidence in support of a synergistic effect of the interaction between u-PA and its cellbound receptor. It needs no argument that our data, generated in this model have to be interpreted with care. However, both bone matrix degrading capacity and previously demonstrated high expression of u-PA and u-PAR in synovial tissue of patients with erosive arthritis support a pathophysiological role of the PA system in the development of bone erosions.

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CHAPTER 4

PLASMINOGEN ACTIVATOR MEDIATED CARTILAGE DEGRADATION BY HUMAN RHEUMATOID FIBROBLAST-LIKE SYNOVIOCYTES; INHIBITION OF PLASMIN ACTIVITY BY DIRECT GENE TRANSFER AS A POSSIBLE INTERVENTION ROUTE

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Submitted

SUMMARY

Proteolytic destruction of cartilage in rheumatoid arthritis (RA) is believed to be mediated, at least in part, by the plasminogen activation (PA) system. We investigated the involvement of this enzyme system in the degradation of articular cartilage by RA synovial fibroblasts (RSFs) *in vitro*, and studied whether interference with the PA system can be used as a possible therapeutic approach to prevent cartilage destruction.
Human RSFs, isolated from RA synovial tissue, were cultured on a ^{3}H-proline labeled cartilage matrix in the presence of plasminogen (plg). Degradation of cartilage matrix was measured by the release of ^{3}H-proline. The capacity of the PA system to degrade intact cartilage was studied using cultures of RSFs with explants of whole bovine articular cartilage in the presence and absence of plg and aprotinin. Degradation was followed by the release of hydroxyproline (Hyp). Finally, the RSFs were infected with an adenovirus encoding the plasmin inhibitor aprotinin, targeted to the urokinase receptor (u-PAR), to investigate a possible inhibiting effect on cartilage degradation.
In the presence of plg, rheumatoid synovial fibroblasts degraded the cartilage matrix up to 65%, whereas in the absence of plg, no degradation was observed. Addition of the plasmin inhibitor aprotinin strongly inhibited matrix degradation. Plg activation was achieved mainly by u-PA, as antibodies against u-PA, but not against t-PA inhibited the cartilage matrix degradation. Culturing RSFs on intact cartilage in the presence of plg resulted in a significant increase of Hyp release, which could be totally inhibited by aprotinin.
Finally, degradation of cartilage matrix could be inhibited by infection of the synoviocytes with the adenovirus vector encoding u-PAR binding aprotinin.
Cartilage degradation by human RSFs *in vitro* is mediated by the PA system. This favors the concept that this enzym system contributes to cartilage destruction in RA.
The reduction of cartilage degradation by inhibition of plasmin by either an exogenous plasmin antagonist or expression of a plasmin inhibitor at the cell surface provides perspectives for the development of a new joint protective therapeutic strategy.

INTRODUCTION

Destruction of articular bone and cartilage leading to funtional loss is a major consequence of rheumatoid arthritis (RA) (1). The hyperplastic inflammatory synovial tissue, degrades and invades articular cartilage and bone by the release of proteolytic enzymes (2).
Although all classes of proteolytic enzymes seem to be involved in tissue destruction, cartilage destruction has mainly been attributed to matrix metalloproteinases (MMPs) and serine proteinases (1). Among the serine proteinases, plasmin and the plasminogen activators (PAs) may be of specific interest. Plasmin has the capacity to degrade a wide

variety of extracellular proteins. In addition, plasmin is able to activate latent forms of collagenase and stromelysin (3,4).
Clinical and experimental observations suggest the involvement of the PA system in rheumatoid joint destruction. A positive correlation between urokinase plasminogen activator (u-PA) and plasminogen activator inhibitors (PAI-1 and PAI-2) concentrations in synovial fluid (SF), and markers of clinical disease activity and radiological destruction has been reported (5-7).
The difference between rheumatoid SF and corresponding plasma antigenic levels of u-PA and PAI-1 indicates local production inside the inflamed joint (8). In contrast to u-PA and PAI-1, the concentration of t-PA has been found to be decreased or unchanged in rheumatoid SF when compared with corresponding plasma levels and non-arthritic SF (9).
The observations made in SF are in line with those in synovial tissue. Extracts of RA synovial tissue were found to contain increased amounts of u-PA, u-PA receptor (u-PAR), PAI-1 and PAI-2, compared with osteoarthritis or normal synovial tissue, whereas t-PA levels were reduced (10,11). Immunohistochemically, these components are mainly located in the hyperplastic lining area of synovial tissue. Synovial macrophages and RSFs are the two major cell types found in this area, which is generally believed to be the invasive front (12). RSFs have been shown to degrade and invade articular cartilage (13). These cells not only show increased production of u-PA (14,15), but also, besides u-PAR expression, show expression of novel proteins involved in plasminogen (plg) binding, which are absent in normal synovial fibroblasts (16). These findings imply involvement of the PA system as part of changing phenotypes of fibroblasts in the rheumatoid joint.
Further evidence for a role of the PA system in joint destruction comes from *in vitro* experimental data. Degradation of cartilage explants by stimulated resident chondrocytes has been shown to be mediated by the PA system (17-19). We previously demonstrated that breakdown of a radiolabeled bone matrix can be achieved by mouse cells transfected with human u-PA. Co-cultivation of u-PA and u-PAR expressing cells resulted in a marked enhancement of matrix degradation indicating the functional importance of the interaction between u-PA and its receptor (20).
Recently, it was found that tranexamic acid, an inhibitor of plg activation, reduces collagen pyridinoline crosslinks excretion in both experimental arthritis and rheumatoid arthritis (21). As such, this observation not only supports the involvement of the PA system in the destructive phase of arthritis, but may also suggest a beneficial effect of therapeutic strategies directed against inhibition of matrix proteolysis. The cells, enzymes and mechanisms involved in cartilage erosion need to be thoroughly understood to open new perspectives for the development of treatment of the destructive phase of the disease.
The objective of this study is to investigate whether the PA system is involved in the capacity of human synovial fibroblasts to degrade articular cartilage *in vitro* and to assess whether plasmin inhibition leads to reduction of the matrix degradation.

MATERIAL AND METHODS

Isolation and culture of synovial fibroblasts

Synovial tissue was obtained with informed consent from patients with active RA who required joint surgery. RSFs were isolated by digestion the synovial tissue in bacterial collagenase type 2 (Worthington Biochemical Corporation, New Jersey, 5 mg/g synovial tissue, wet weight) at 37 °C during 4 hours and washed thoroughly with PBS to remove collagenase. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)\Ham's F-12 medium (Gibco Life Technologies, Inc. Gaiterburg, MD) supplemented with 10% (v/v) new born calf serum (NBCS), 10% (v/v) human serum (HS), 100 U/ml penicillin and 100 µg/ml streptomycin (Boehringer Mannheim, Mannheim, Germany) at 37 °C in a humidified atmosphere of 5% carbondioxide. RSFs up to the 3rd passage were used for degradation experiments. Production of u-PA was measured with an enzyme-linked immuno-assay according to Koolwijk et al. (23). T-PA antigen was determined using a commercially available enzyme immunoassay kit (Thrombonostika t-PA; Organon Teknika, Turnhout, Belgium (24)).The presence of u-PAR on the RSFs was determined according to Nielsen et al. (25). Essentially, this method involves the moderate acid treatment of cells to remove endogenous bound u-PA, incubation of cell lysates with radiolabeled aminoterminal fragment of Di-isopropylfluorphosphate (DFP) treated u-PA, ^{125}I-uPA, and crosslinking of the u-PAR/ ^{125}I-u-PA complex with disuccininidyl suberate. u-PAR/ ^{125}I-u-PA complex is visualized by autoradiography after gel electrophoresis (26).

Production of radiolabeled cartilage matrix

Chondrocytes were cultured in alginate beads as described previously (27).
These chondrocytes retain their cartilage-specific phenotype for at least 8 months. 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 (28).
Chondrocytes were obtained from bovine metacarpophalangeal articular cartilage. Cartilage slices were digested by bacterial collagenase type 2 solution (0,1% (w/v) in DMEM supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, 5 mg collagenase/g cartilage, wet weight) at 37 °C overnight. After filtration (filterroom NPBI, Emmer-Compascuum, Netherlands) and 3 times washing in DMEM, chondrocytes were suspended in a low viscosity (1.2%) alginate gel (Kelco, San Diego, CA) at a density of $4x10^6$ cells/ml. Beads were formed by dropping the gel into a 100 mM $CaCl_2$ solution and were subsequently incubated at room temperature for 20 minutes. The embedded chondrocytes were cultured in DMEM supplemented with 10% (v/v) NBCS, 50 µg/ml ascorbic acid, penicillin (100 units/ml) and streptomycin (100 µg/ml) at 37 °C in a humidified atmosphere of 5% carbon dioxide. After culturing for 4 days, 0.25 µCi/ml ^{3}H-proline (^{3}H-pro)(Amersham International, Amersham, UK) was added to fresh medium to

be incorporated into the matrix (mainly collagen). The ^{3}H Pro incorporated in the matrix was predominantly found in collagen , (^{3}H Hyp and ^{3}H Pro) as determined by amino acid HPLC analysis (29) after acid hydrolysis of the matrix.
Medium was changed twice weekly. After culturing for 28 days, the alginate gel was suspended in 55 mM sodium citrate to solubilize the beads and release the synthesized matrix. The solution was centrifuged (750 g, 6 minutes) and the matrix pellet was dissolved 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.

Culture of bovine articular cartilage explants
Bovine metacarpophalangeal joints were acquired from a local abattoir immediately after the sacrifice of cows (1-2 years old). The specimens were thoroughly washed with 70% (v/v) ethanol and 2% (w/v) Halamid-D (AKZO NOBEL, Wijk bij Duurstede, The Netherlands). Articular cartilage was removed aseptically from the joint, washed twice with PBS and prepared as slices with a wet weight of approximately 30 mg. The cartilage slices were incubated in DMEM at 37 °C in a humidified atmosphere of 5% (v/v) carbon dioxide. Each culture consisted of one piece of cartilage in 0.5 ml of medium.
To obtain cartilage tissue without viable chondrocytes, explants were frozen in liquid nitrogen and thawed, repeating this procedure 3 times.

Degradation of ^{3}H-pro labeled cartilage matrix by synovial fibroblasts
Radiolabeled cartilage matrices were slowly thawed at room temperature in M-199 culture medium (Bio Whittaker, Verviers, Belgium) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. To measure matrix degradation, RSFs were seeded (2 x 10^{4} /cm^{2}) onto the matrix (figure 1) and in culture medium containing 5% (v/v) HS, 5% (v/v) NBCS, 100 U/ml penicillin, 100 µg/ml streptomycin and supplemented with extra plg (0.15 µM) to prevent an effect of variations in plg content of NBCS and HSA. In order to allow both stimulating and inhibiting effects, we chose a maximal degradation of approximately 60%. Such levels were reached after 72 hours of incubation (at 37 °C, humidified air containing 5% (v/v) carbon dioxide).
The following inhibitors were added to different wells: monoclonal anti-human u-PA IgG (30) 15 µg/ml, polyclonal anti-human t-PA (31) 15 µg/ml, and aprotinin (Trasylol®), a potent inhibitor of plasmin at a final concentration of 100 U/ml.
After 72 hours of culture, the supernatant medium was removed and residual matrices were dissolved in 0.1 M NaOH. The release of radioactivity in the medium and the amount of radioactivity in the matrix was counted by a liquid scintillation analyzer (Tri-Carb 1900 CA, Packard). The percentage of matrix degradation was calculated as:
(dpm [medium]/(dpm [medium] + dpm [matrix])) x 100%.

Degradation of cartilage explants

RSFs were seeded in culture wells (2 x 10^4 /cm^2 , 0.5 ml medium) containing explants of bovine articular cartilage in M-199 medium supplemented with 10% (v/v) NBCS, 10% (v/v) HS, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were cultured for 72 hours at 37 °C with air containing 5% (v/v) carbon dioxide, in the presence or absence of aprotinin at a final concentration of 100 U/ml. Plg (0.15 µM) was added after 24 hours. Degradation of the cartilage was assessed by measuring Hyp release into the culture medium by HPLC (32), expressed as pmol/mg cartilage and corrected for background release.

Infection of synovial fibroblasts with ATF.BPTI encoding adenovirus

Directly after seeding the synovial fibroblasts onto the ^{3}H proline labeled cartilage matrix in culture medium containing 5% NBCS, they were infected with the replication deficient adenovirus AdATF.BPTI at concentration 5x10^7 Pfu/ml and 1x10^8 Pfu/ml and with AdLacZ (1x10^8 Pfu/ml) as a control. The AdATF.BPTI virus encodes a hybrid protein, designated ATF.BPTI, that consits of the aminoterminal fragment (ATF), the receptor binding part, of u-PA, to which bovine pancreatic trypsin inhibitor (BPTI), also known as aprotinin or Trasylol® is attached. This hybrid protein can bind to the u-PAR on the cell surface and inhibits plasmin activity in the direct pericellular environment.
Three hours after infection the culture medium was refreshed. Finally, 24 hours after infection plg was added and the cartilage matrix degradation was monitored as described.

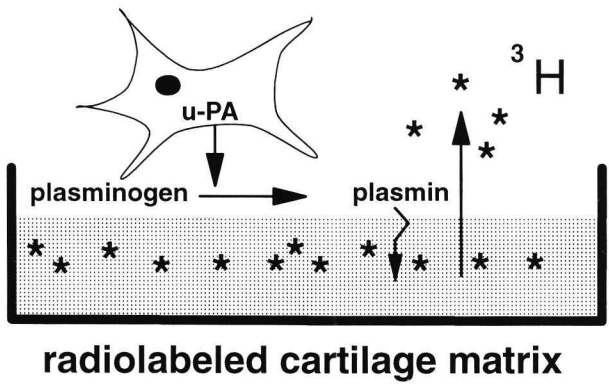

Figure 1. A schematic diagram illustrating the culture of rheumatoid synovial fibroblasts on radiolabeled cartilage matrix, in the presence of plasminogen.

Calculations and statistical analysis
All measurements 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 *Students t-test* for independent samples. All calculations were performed with the standard software package SPSS.

RESULTS

Rheumatoid synovial fibroblasts degrade radiolabeled cartilage matrix by u-PA mediated plasminogen activation.
After 96 hours of culture, RSFs produced u-PA to a level of 1.2 ng/ml (figure 2), whereas t-PA production was less than 0.05 ng/ml (data not shown).
Culturing synoviocytes on top of the cartilage matrix in the presence of plg, resulted in an increased release of radiolabel of approximately 60% after 72 hours. Only a small release of ^{3}H-pro was observed in the absence of cells.The degradation of ^{3}H-pro labeled matrix components could be totally blocked by aprotinin, indicating the involvement of active plasmin. The formation of plasmin was achieved by u-PA, as antibodies to u-PA, in contrast to t-PA antibodies, significantly inhibited the ^{3}H-pro release (figure 3).

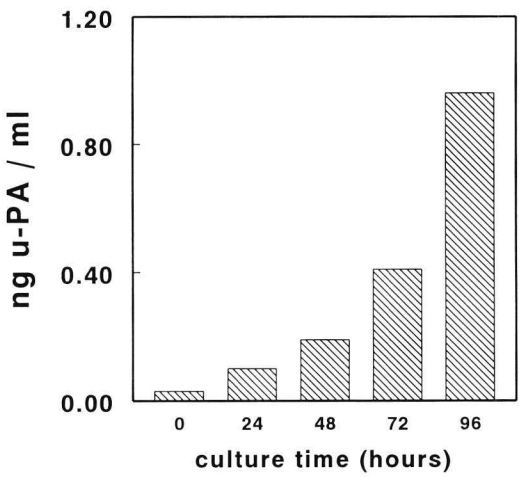

Figure 2. u-PA production by human rheumatoid fibroblast-like synoviocytes during 96 hours of culture.

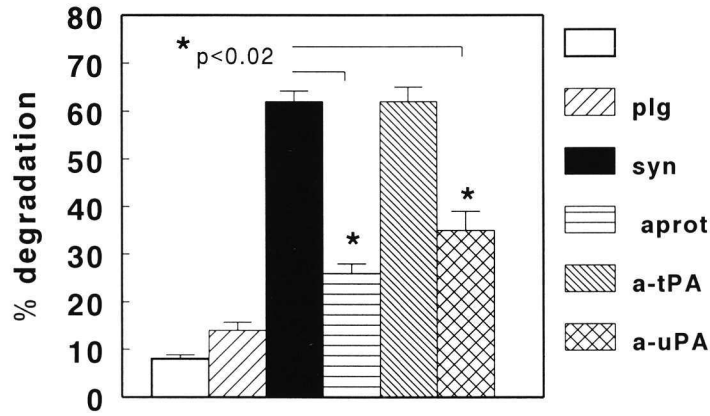

Figure 3. In vitro degradation of a ^{3}H-pro labeled cartilage matrix in the absence and presence of plasminogen (plg;0.15 μM), and cultured with rheumatoid synovial fibroblasts (syn) in the presence of plg during 72 hours. The effects of the addition of aprotinin (aprot;100 U/ml), anti t-PA (15 μg/ml), and anti u-PA antibodies (15 μg/ml) to the synoviocyte cultures are shown. Results are given as the mean and standard error of the mean ^{3}H-pro release in the medium as a percentage of the total radioactivity (n=3).

The PA system is involved in the degradation of articular cartilage by synovial fibroblasts in vitro.

RSFs were cultured with explants of intact bovine articular cartilage to investigate whether the PA system is also involved in the degradation of intact cartilage. Degradation of cartilage was assessed by measuring the release of Hyp from the cartilage explants in the presence and absence of plg, and aprotinin.

RSFs increased the release of Hyp with approximately 20 pmol/mg. Supplementation of the culture system with 0.15 μM plg resulted in a further release of Hyp-containing cartilage components towards a level of approximately 35 pmol/mg. This matrix degrading effect was, at least partly, due to activation of plg by the cells, as addition of the plasmin inhibitor aprotinin decreased Hyp release towards background level (figure 4).

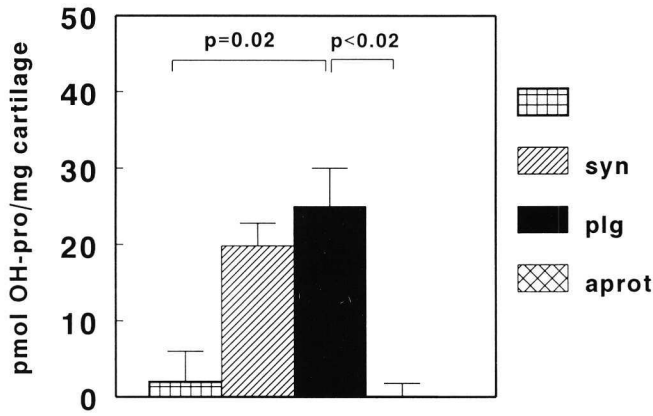

Figure 4. In vitro degradation of explants of intact bovine articular cartilage by human rheumatoid synovial fibroblasts (syn) after 72 hours of cultures as measured by the hydroxyproline (Hyp) release above background level. The effects of supplementation of plasminogen (plg;0.15 μM) and aprotinin (aprot;10 U/ml) are shown. Results are given as the mean and standard error of the mean Hyp release in the medium above background level(n=3).

Infection of human RA synovial fibroblasts with adenovirus encoding for ATF-BPTI significantly reduces PA mediated cartilage matrix degradation.

Incubation of the RSF-cell lysates with ^{125}I-ATF, resulted in formation of u-PAR/ ^{125}I-ATF complexes (data not shown), confirming the expression of u-PAR by RSFs (16).

Culturing RSFs with radiolabeled cartilage matrix in the presence of plasminogen resulted in matrix degradation due to plg activation.

In order to investigate whether targeted inhibition of plasmin at the site of its activation at the cell surface has functional consequences, we infected the cells with an adenovirus encoding ATF.BPTI and assessed the effect on ^{3}H-Pro release after 72 hours culture. Low dose AdATF.BPTI ($5x10^{7}$ pfu/ml) resulted in a significant reduction of radiolabel release. High dosage of ATF-BPTI encoding adenovirus ($1x10^{8}$ pfu/ml) further decreased degradation towards baseline level, a blocking effect that could also be also accomplished by aprotinin. Infection with high dose AdLacZ ($1x10^{8}$ pfu/ml) virus had no significant effect on ^{3}H-pro release (figure 5).

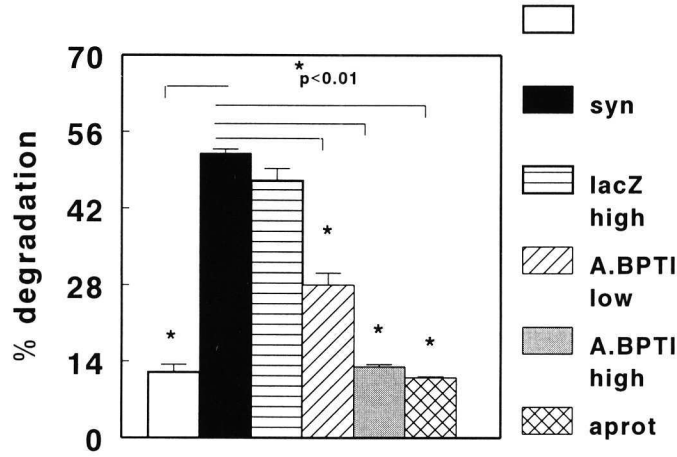

Figure 5. Degradation of ^{3}H-pro labeled cartilage matrix by rheumatoid synovial fibroblasts (syn) cultured during 72 hours on top of this matrix in the presence of plasminogen (plg;0.15 μM). The effects of infection with the control virus LacZ, both high low dose (5x10 ^{7} pfu/ml) and high dose (1x10 ^{8} /ml) adenovirus encoding ATF.BPTI, and aprotinin (aprot;10 U/ml) are shown. Results are given as the mean and standard error of the mean ^{3}H-pro release in the medium as a percentage of the total radioactivity (n=3).

DISCUSSION

Cartilage destruction in RA is partly accomplished by the release of proteinases by activated synovial cells, chondrocytes, and inflammatory cells (3). Plasmin could play a pivotal role in this process, since it possesses the capacity to degrade matrix proteins directly as well as indirectly by activation of latent MMPs (3,4, 33). Its precursor plg, is abundantly present in plasma and synovial fluid in a concentration of approximately 1-2 μM. In RA, its main activator is u-PA, which is produced by a wide variety of cells within the joint. (34-36). The possible involvement of the PA system in cartilage degradation has been illustrated by the capacity of stimulated chondrocytes to express u-PA activity and to degrade cartilage matrix in the presence of plg (17-19).
RSFs have been shown to invade and destroy articular cartilage (12,13). One of the mechanisms involved could be the activation of plg by production and release of plg

activators localized at the surface of these cells (14-16). In the present study we demonstrate that human RSFs are able to degrade cartilage in the presence of plg. Since the inhibitor of plasmin, aprotinin strongly reduced the cartilage degradation, plg activation must be a pathway by which this degradation was achieved. It appears that u-PA was the dominant plg activator since anti-u PA antibodies, but not anti-tPA antibodies significantly reduced the release of cartilage matrix components. The predominance of u-PA over t-PA in plg activation implicated in cartilage breakdown by RSFs is well in accordance with the specifically increased expression of u-PA and the reduced levels of t-PA in the RA joint (8-11).

In this study, u-PA mediated plg activation resulted in the release of radiolabeled proline from the cartilage matrix, and of hydroxyproline from explants of whole articular cartilage. These findings indicate collagenolytic and gelatinolytic activity. Since plasmin has only moderate capacity to degrade denatured collagen and gelatin, activation of pro-MMPs was probably involved in the cartilage degradation we observed. Several lines of evidence suggest that plasmin may play a substantial role in cell-mediated collagen degradation. It has been reported that chondrocytes cultured on a collagen film only degraded the collagen in the presence of plg, an effect that could be inhibited by tissue inhibitor of MMPs (33). Furthermore, it has been reported that binding of both plg and u-PA to the cell surface results in activation of pro-MMP2 and pro-MMP 9, whereas soluble plasmin was shown to degrade gelatinase (37). Recently, evidence was obtained for a plg dependent, pro-MMP activation cascade leading to cartilage collagen degradation (38). In our culture system, RSF culture medium supplemented with NBCS, and the cartilage matrix might have been sources of pro-MMPs.

We found that infection of RSFs with an adenovirus encoding ATF.BPTI strongly reduced the degradation of the cartilage matrix accomplished by these cells. This inhibiting effect may be attributed to several mechanisms. Firstly, the expression of BPTI by RSFs could result in reduction of pericellular proteolytic activity by inhibition of plasmin.

Plg activation has been shown to be strongly enhanced at the cell surface when it is simultaneously present in close proximity to receptor-bound u-PA (39). Cell bound plasmin in this situation, protected from its natural inhibitors (40), can subsequently activate latent u-PA (41). It can be envisaged that the hybrid protein ATF.BPTI localizes the plasmin inhibitor on the cell surface at the specific site where plg activation takes place, leading to disturbance of the accellerating activation cascade.

Previously, we have demonstrated that interaction between u-PA and u-PAR results in enhancement of matrix degradation (42,43). The competitive inhibition of u-PA binding with u-PAR by ATF, could be a second mechanism to prevent efficient plasmin formation and subsequent matrix degradation.

Intervention with the PA system may provide a potential way to reduce joint destruction in arthritis. Chondroprotective effects have been demonstrated of intraarticularly administered aprotinin, tranexamic acid, an inhibitor of plg activation, and protease nexin-1 (44-47).

In patients with RA, clinical improvement was found after intra-articular injection of urinary trypsin inhibitor, which probably inhibited u-PA mediated plg activition (48). Recently, we found that oral administration of tranexamic acid to patients with RA significantly reduced the excretion of collagen degradation products (21).
In conclusion, cartilage degradation by human rheumatoid synovial fibroblasts *in vitro* is mediated by the plasminogen activation system. These results and the previously demonstrated increased expression of the PA system in rheumatoid joints favor the concept that the PA system contributes to cartilage destruction in RA. The inhibition of cartilage degradation by a plasmin inhibitor, expressed by degrading cells, provides perspective for the development of new joint protective therapeutic tools.

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CHAPTER 5

TRANEXAMIC ACID, AN INHIBITOR OF PLASMINOGEN ACTIVATION, REDUCES URINARY COLLAGEN CROSS-LINK EXCRETION IN BOTH EXPERIMENTAL AND RHEUMATOID ARTHRITIS

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SUMMARY

The plasminogen activation system is one of the enzyme systems held responsible for bone and cartilage degradation in rheumatoid arthritis (RA). In this study, we evaluated the effect of tranexamic acid (TEA), an inhibitor of plasminogen activation, on urinary collagen crosslink excretion and radiological joint damage in rat adjuvant arthritis (AA) and on urinary collagen crosslink excretion in patients with rheumatoid arthritis.

In the animal study, adjuvant arthritis was induced in male Lewis rats. From day 7 onward, high dose TEA (500 mg/kg bodyweight, once daily) or placebo was administered orally. Study groups consisted of TEA treated normal rats (C+TEA), placebo treated normal rats (C+plac), AA rats treated with TEA (AA+TEA) or with placebo (AA+plac). To monitor joint destruction, urinary collagen crosslink excretion (pyridinoline, HP; deoxypyridinoline, LP) was measured by HPLC at day 14 and 21. Radiological evaluation of joints was performed at day 21.

In the patient study, TEA was administered to nine patients with RA as adjuvant medication ($\sim$20 mg/kg bodyweight, 3 times daily) for 12 weeks. Urinary HP and LP excretion levels were measured before and during TEA treatment, and 4 weeks after cessation of TEA treatment.

In AA+TEA rats, a significant reduction of HP and a tendency towards reduction of LP excretion was found compared with AA+plac rats ($p < 0.05$), at day 14, whereas HP/LP ratio did not change. No difference was observed in HP, LP excretion, HP/LP ratio and radiologic damage score between the TEA and placebo treated AA rats at day 21.

In RA patients a significant reduction of HP and LP excretion was found during the TEA treatment period ($p < 0.05$). After cessation of TEA treatment, HP and LP excretion increased towards baseline levels. No effect on disease activity was observed.

The plasmin antagonist TEA reduced the excretion of collagen pyridinoline crosslinks in both experimental and rheumatoid arthritis. As such this study not only supports the involvement of the plasminogen activation system in the destructive phase of arthritis, but also suggests a beneficial effect of therapeutic strategies directed against inhibition of matrix proteolysis.

INTRODUCTION

In rheumatoid arthritis (RA), joint destruction is, at least in part, due to the action of excessive amounts of proteolytic enzymes produced at the site of inflamed synovial tissue. One of the enzyme systems held responsible for bone and cartilage destruction in RA is the plasminogen activation (PA) system (1-3). The key enzyme of the PA system, plasmin, is able to degrade extracellular matrix directly (4) as well as activate latent matrix metalloproteinases (MMPs) (5). Increased expression of urokinase plasminogen

activator (u-PA) and its receptor (u-PAR) in synovial tissue of patients with RA (6), as well as bone matrix degrading capacity of the PA system (7) support a pathophysiologic role of this enzyme system in the development of joint damage in RA.
In this study, the plasmin antagonist tranexamic acid (TEA) was selected to assess its possible therapeutic effects in destructive inflammatory arthropathy. This widely used and registered drug has only mild adverse effects. After oral administration, TEA is distributed over the extracellular and intracellular compartments and diffuses rapidly into the synovial fluid where concentrations comparable to serum are reached (8,9).
The biological half life in joint fluid is about 3 hours. Since the drug is rapidly cleared, it has to be given at short intervals to maintain therapeutic levels (10). Its anti-plasmin activity is probably based on competitive blocking of plasminogen binding sites (11).
In an attempt to find more evidence for a pathogenic role of the PA system in inflammatory joint destruction, we first studied the effect of TEA administration on urinary collagen crosslink excretion and radiologic damage in the adjuvant model of chronic arthritis (AA) in rats. Based on these findings, the study was extended to an open label study in patients with erosive RA.
Collagen crosslinks pyridinoline (HP) and deoxypyridinoline (LP), excreted in urine, were used as the main parameters to monitor joint destruction. LP is considered an appropriate marker of bone collagen degradation, whereas HP reflects degradation of collagen that predominates in bone and cartilage (types I and II). Both types of collagen crosslinks are excreted in higher rates in RA and their urinary excretion provides an estimate of joint destruction in arthritis (12,13).

ANIMALS, PATIENTS, MATERIALS AND METHODS

Animal study

Animals and induction of adjuvant arthritis. Male Lewis rats, aged seven to eight weeks, weighing at least 190 g were maintained in accordance with the Long Island Jewish Medical Hospital Animal Care and Use Committee. Adjuvant arthritis was induced by a single intradermal injection distal to the base of the rat tail, containing 50 µl of a 6 mg/ml suspension of heat killed mycobacterium tuberculosis in light mineral oil.

Study design. Animals were randomly allocated to 4 experimental groups. Study groups consisted of placebo treated control rats (C+plac; n=4), control rats receiving 500 mg/kg TEA (C+TEA; n=5), placebo treated treated adjuvant arthritis rats (AA+plac; n=9) and AA rats treated with TEA (AA+TEA; n=9). Starting on the 7th day after induction of adjuvant arthritis, a single daily dose of 500 mg/kg body weight TEA (Sigma Chemical Co, St. Louis, USA) suspended in 2% carboxymethylcellulose, or placebo was administered by oral gavage.

Laboratory assessment. At day 14 and 21 after induction of adjuvant arthritis, animals were kept in metabolic cages to collect 24-hours urine. Urinary collagen crosslinks HP and LP were measured by high performance liquid chromatography (HPLC) after hydrolysis of urine samples in 6M HCl (14) and expressed as nmol/mmol creatinine.

Radiological assessment. At the end of the study (day 21), the animals were sacrificed. The right hind limbs were amputated above the knee joint, fixed on cardboard, frozen and stored at -20 °C until they were radiographically imaged (lateromedial view) on Kodak (Rochester, NY), X-omat MA film with exposure time 320 ms. Radiological evaluation of joint damage was performed by three observers, unaware of the treatment. Abnormalities in ankle and foot were graded as: 1 = no lesion, 2 = slight osteoporosis, 3 = pronounced osteoporosis and slight erosions, 4 = massive osteoporosis, erosions and disappearance of joint structures.

Patient study

Patient population. Patients with RA fulfilling the 1987 criteria of the American Rheumatism Association (15), with a disease duration of less than 10 years were included in the study. Further criteria for entry were erosive arthritis of at least one joint, as judged by radiological examination aa well as active disease, defined by the presence of 6 or more swollen joints, and at least 2 of the following criteria: (1) the presence of 9 or more joints painful on motion or tender on pressure; (2) morning stiffness lasting at least 45 minutes; (3) an ESR (erythrocyte sedimentation rate) of at least 28 mm/h.

Second-line therapy had to be unchanged for at least 6 months prior to, and during the trial. Corticosteroid usage was permitted, provided the daily dose did not exceed 7.5 mg

at entry and had been unchanged for at least 3 months. Intra-articular injections with corticosteroids were not allowed for one 1 month prior to the trial and during the course of the trial.
Patients with a history of thromboembolic disease or patients using drugs acting on haemostasis, except NSAIDs (non steroidal anti-inflammatory drug), were excluded. Other exclusion criteria were macroscopic hematuria from upper urinary tract, pregnancy or lactation and renal function impairment.
Study design. This was a 12-week open label trial. Nine consecutive patients, attending the outpatient clinic department of the University Hospital, Leiden and fulfilling the eligibility criteria received TEA as adjuvant medication after their informed consent. Clinical and laboratory assessment was performed at weeks -2, -1, at study entry (week 0), at 4, 8 and 12 weeks during the study. The follow up period lasted 4 weeks after discontinuation of the trial medication to document a possible rebound effect. The study was approved by the medical ethical committee of the hospital.
Drug administration. The trial dosage for TEA (cyklokapron®, tablets of 0.5 gram, Pharmacia Upjohn BV, The Netherlands) was 1.5 gram p.o., 3 times daily, during 12 weeks. Compliance was checked by pill counting. TEA was given in addition to traditional treatment of the patient with a DMARD, NSAID and/or corticosteroids, which were kept constant for the duration of the study. The only additive analgesic allowed during the study was paracetamol.
Clinical assessment. All patients were examined by the same investigator. Disease activity was determined according to a modified disease activity score using twenty-eight joint counts (16) and by measurement of the erythrocyte sedimentation rate (ESR).
Adverse effects. Patients were interviewed about side effects at each visit. In case of side effects, the daily dosage of TEA was reduced until adverse effects diminished.
Laboratory assessment. HP and LP were quantitated in morning samples of hydrolyzed urine by HPLC and expressed as nmol/mmol creatinine.
Statistical analysis. Data are presented as mean $\pm$ standard error of the mean (SEM). In the animal study, differences in crosslink excretion between experimental groups were analyzed with the non-parametric Wilcoxon's rank sum test for unpaired data. In the patient study, the intra-individual differences in the efficacy parameters were analyzed with the non-parametric Wilcoxon's rank sum test for paired data. Differences were considered significant when $p < 0.05$. The relationship between crosslink excretion and radiologic damage was determined by linear regression.

RESULTS

Animal study

Study course data and collagen crosslink excretion

In control animals, urinary levels of pyridinoline crosslinks and the HP/LP ratio remained constant during the study ($p > 0.05$, day 14 vs day 21. Fig. 1). In the AA group however, HP and the HP/LP ratio increased significantly from day 14 to day 21 (Fig. 1 A, C). LP excretion rates showed a tendency towards increase, which was not statistically significant (Fig. 1, B)

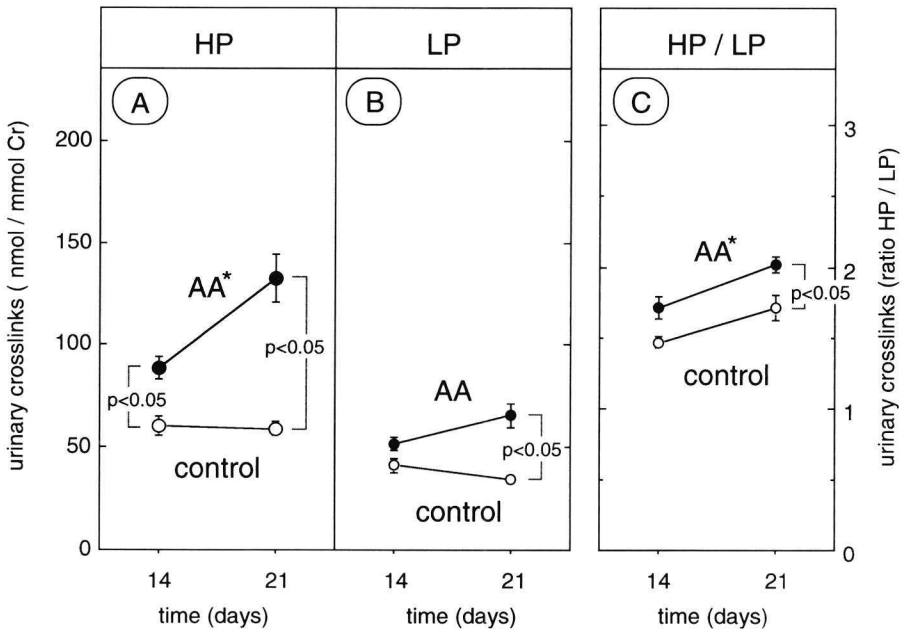


Figure 1. In the adjuvant model for arthritis, urinary excretion of HP, LP, and the HP/LP ratio in control animals is compared with arthritic (AA) animals, irrespective of their treatment. Data are presented as mean ± standard error of the mean.
(Statistically significant difference; $p < 0.05$, Wilcoxon's rank sum test).*

During the early phase of arthritis, at day 14, HP levels were significantly elevated in AA rats compared to control animals. At day 21, when the disease had developed to fulminant arthritis, not only HP but also LP levels were significantly elevated in AA when compared to control animals.

Effect of TEA on urinary pyridinoline excretion
At day 14, in control animals, TEA did not affect the excretion rate of HP, LP or the HP/LP ratio (Fig. 2).
In the AA animals treated with TEA, excretion rates of HP were significantly reduced in comparison with the placebo-treated arthritic animals (AA+TEA vs AA+plac: $p<0.05$, Fig. 2). LP excretion rates showed a tendency towards reduction, which was not statistically significant (AA+TEA vs AA+plac: $p>0.05$, Fig. 2). Crosslink excretion levels in TEA treated arthritic rats were reduced to control levels (AA+TEA vs C or C+plac: $p>0.05$, Fig. 2). HP/LP ratio remained unchanged in all 4 experimental groups.
At day 21, no effect of TEA was observed (AA+TEA vs AA+plac: $p>0.05$).

Radiologic joint destruction and its relation with pyridinoline excretion
Radiologic evaluation revealed no abnormalities in non-arthritic control rats. Severe joint damage was observed in both AA+plac and AA+TEA rats, without significant difference between the two treatment groups.
At day 14, HP and LP excretion levels were proportional to the radiologic joint damage that was observed one week later ($p<0.05$, Fig. 3). Stronger correlations were obtained at day 21: HP ($r=0.90$) and LP ($r=0.88$) levels increased linearly with radiologic damage up to phase ≤ 2 (on a scale of 1-4; $p<0.001$). In cases of excessive radiologic damage (score > 2), pyridinoline excretion rose to maximal values that were no longer related to radiological damage (Fig. 3, "shaded" curve).

Patient study
All patients completed the study and full compliance was obtained. One patient reported diarrhea that diminished after reduction of the TEA dose from 1.5 to 1 gram daily.
The clinical efficacy parameters of all patients remained constant during the study.
No significant change was observed in modified disease activity score and ESR.Rates of urinary collagen crosslink excretion at week -2, -1 and 0 were identical ($p>0.05$). Therefore, the mean urinary concentration of the crosslinks at week -2, -1 and 0 was taken as the baseline value to compare with the excretion during and after TEA treatment.
After 4 weeks of TEA treatment a small but statistically non significant reduction in HP and LP excretion was observed. After 8 weeks and 12 weeks of treatment a statistically significant reduction was observed in HP and LP excretion ($p<0.05$, Fig. 4 A,B).

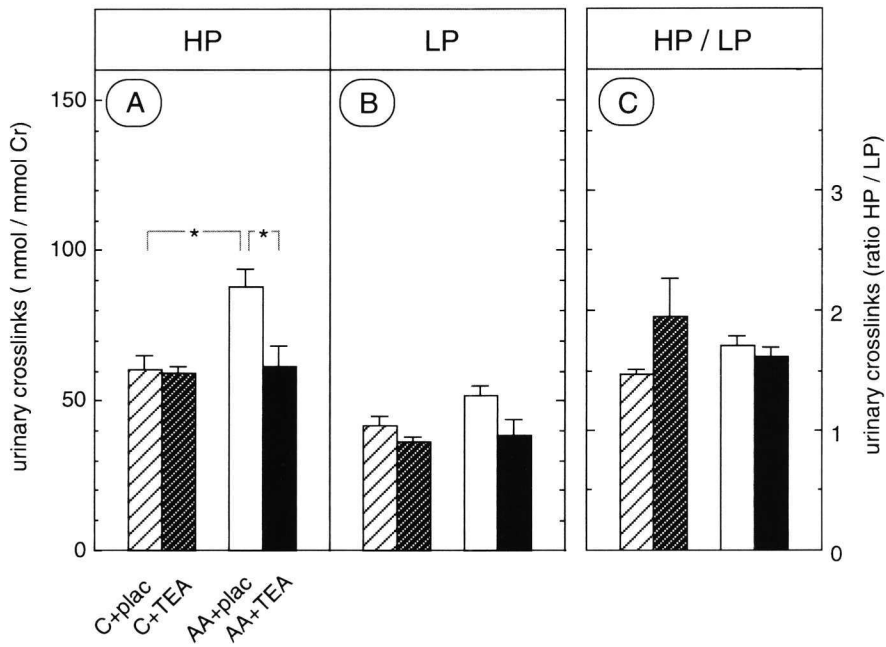


Figure 2. In the adjuvant model for arthritis, urinary excretion of HP, LP, and the HP/LP ratio in placebo or TEA treated control animals is compared with AA rats treated with placebo or TEA treated AA rats, at day 14. Data are presented as mean ± standard error of the mean. (Statistically significant difference; $p < 0.05$, Wilcoxon's rank sum test for unpaired data).*

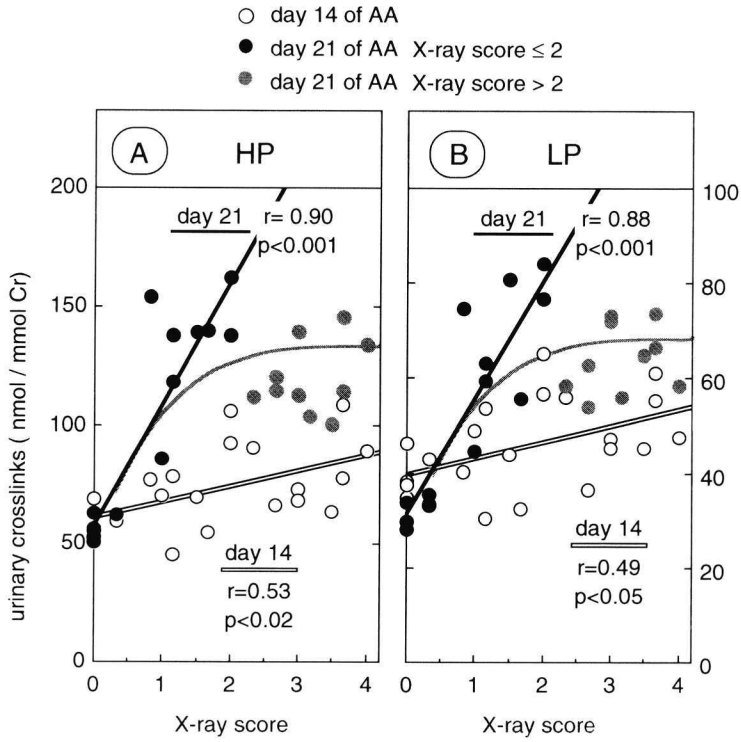
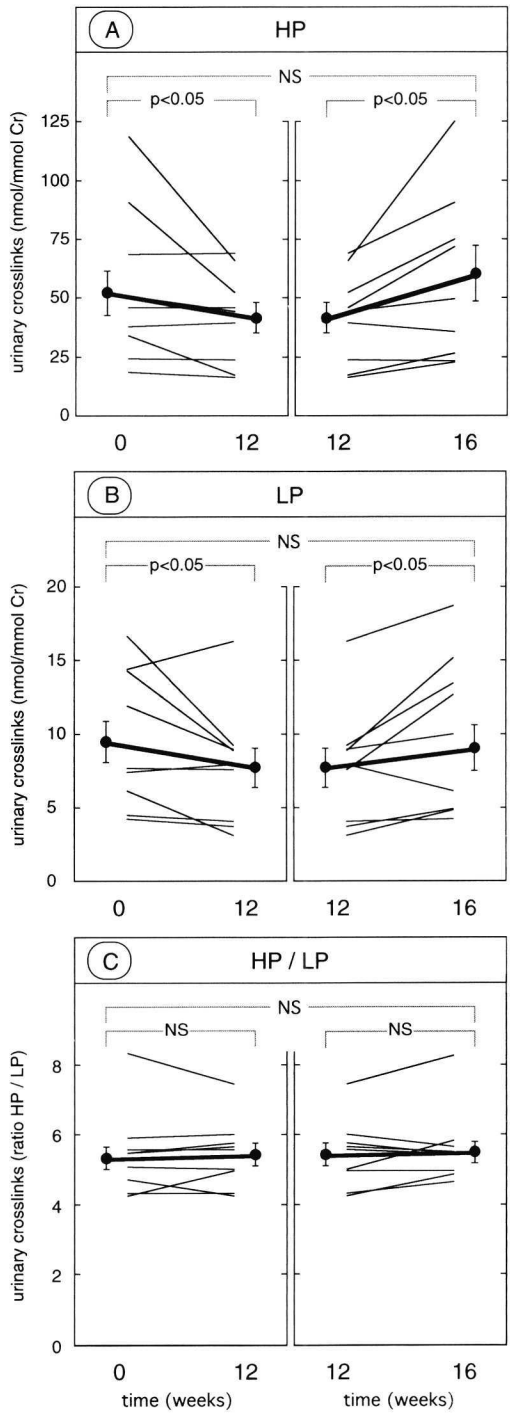


Figure 3. The relationship between HP and LP excretion on the one hand and radiologic damage on the other hand in the adjuvant arthritis model. Correlations were determined at day 14 ("double-lined" curve), at day 21 ("shaded" curve) and at day 21 with radiologic damage up to phase ≤ 2 ("bold" line) (r=linear regression coefficient).

Figure 4. HP, LP excretion and HP/LP ratio in RA patients during a 12 weeks TEA treatment and 4 weeks after cessation of the medication. Data are presented as mean ± standard error of the mean. (Statistically significant difference; $p < 0.05$, Wilcoxon's rank sum test for paired data).


A
HP
NS
p<0.05
p<0.05
urinary crosslinks (nmol/mmol Cr)
125
100
75
50
25
0
0
12
12
16
B
LP
NS
p<0.05
p<0.05
urinary crosslinks (nmol/mmol Cr)
20
15
10
5
0
0
12
12
16
C
HP / LP
NS
NS
NS
urinary crosslinks (ratio HP / LP)
8
6
4
2
0
0
12
12
16
time (weeks)
time (weeks)

DISCUSSION

The main finding of this study is that oral administration of an anti-proteolytic agent results in a decrease of collagen degradation. In the adjuvant model of arthritis, TEA reduced the increased excretion rates of pyridinolines towards control levels early in the disease. In the study with RA patients, TEA decreased pyridinoline excretion after 8 weeks of administration and this suppressive effect disappeared when TEA administration was discontinued. These findings provide further evidence for a pathophysiological role of the PA system in arthritis and are in accordance with previous observations (17,18). High concentrations of urokinase-type plasminogen activator (u-PA) and its inhibitor (PAI) in synovial fluid (19) and increased expression of u-PA, u-PA receptor (u-PAR), PAI-1 and PAI-2 have been demonstrated in synovial tissue of RA patients (6). Rheumatoid synovial fibroblasts show increased production of u-PA (1,20) and u-PAR but also express proteins involved in plasminogen binding which are absent in normal synovial fibroblasts (21). Binding of plasminogen to its cell-bound receptor protects this enzyme from inhibitors, and co-localization of plasminogen and u-PA on the cell surface stimulates plasmin formation (22). This situation may lead to enhancement of extracellular matrix degradation.
The results of the present study are consistent with TEA-mediated competitive interference with the plasminogen-substrate and cellular plasminogen binding sites, resulting in inhibition of plasmin activity and subsequent suppression of pro-MMP activation. This concept is partly based on the observation that TEA inhibited bone matrix degradation by u-PA producing cells in the presence of plasminogen in vitro (7). Beneficial effects of TEA on joint destruction have been demonstrated in animal models of osteoarthritis. Intra-articular administration of TEA prevents loss of proteoglycans from cartilage (17), and intramuscularly administered TEA suppresses the activity of stromelysin as well as collagenase in synovial membrane and reduces cartilage degeneration (18). In the present study we could not demonstrate a joint protective effect at the radiological level. Two explanations may be brought forward. Firstly, the pharmacokinetics of TEA, are not ideal to demonstrate a protective effect on joint destruction in adjuvant arthritis. The drug is rapidly cleared and has to be administered at short intervals to maintain therapeutic levels. Oral administration once daily to rats as used for practical reasons in the present study is unlikely to result in sufficiently high and lasting concentrations of TEA in the joint. Secondly, a highly destructive animal model for arthritis was used, and it can be envisaged that a possible mild protective effect of TEA was masked by severe damage of joints which developed.
Our concept of the use of TEA focuses on joint degradation rather than on an anti-inflammatory effect. The lack of effect on indices of inflammation such as joint symptoms and ESR, and the observed reduction of the excretion of collagen breakdown products not only indicates that a PA antagonist may be useful to reduce joint

destruction, but also supports the idea that joint inflammation and joint destruction are distinct pathophysiological entities (23). Whereas most anti-rheumatic strategies aim to suppress inflammation, inhibition of proteolytic extracellular matrix degradation may also be advantageous to patients with RA. To our knowledge the presented data provide the first evidence of such a beneficial effect at the level of matrix-degrading proteinases. In this respect, TEA, which is a readily available drug with a mild toxicity profile should be considered a lead compound to establish this therapeutic principle.The results of this pilot study merit further research to determine to what extent the reduction in collagen crosslink excretion is clinically relevant.
In conclusion, the inhibitory effect of TEA on pyridinoline excretion further supports a pathogenic role of the plasminogen activation system in destructive joint disease.

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CHAPTER 6

HUMAN GRANZYME B MEDIATES CARTILAGE PROTEOGLYCAN DEGRADATION AND IS EXPRESSED AT THE CHONDRO-SYNOVIAL JUNCTION IN RHEUMATOID ARTHRITIS

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Submitted

SUMMARY

It has previously been shown that the levels of soluble granzyme (Gran)B in synovial fluid and the number of Gran B positive cells in synovial tissue are specifically increased in patients with rheumatoid arthritis (RA). To investigate a possible role of Gran B in cartilage destruction, we assessed its capacity to degrade cartilage and the presence of Gran B positive cells in human rheumatoid synovial tissue at the pannus-cartilage junction.

Gran B was added to radiolabeled cartilage matrices, produced by bovine articular chondrocytes cultured in alginate beads; differential degradation of proteoglycans and collagen was followed by the release of ^{35}S-sulphate and ^{3}H-proline, respectively. Furthermore, Gran B mediated degradation of intact bovine articular cartilage was assessed by the release of sulphated proteoglycans and hydroxyproline into the culture medium and by measurement of the amount of degraded collagen in the cartilage.

The expression of Gran B at the cartilage-pannus junction was studied by immunohistochemistry of metacarpophalangeal joints from RA patients.

Gran B induced degradation of both newly synthesized, radiolabeled proteoglycans in cartilage matrices and the resident proteoglycans of the cartilage explants, whereas no effect on degradation of collagen was found. Gran B positive cells were found at the cartilage-pannus junction of metacarpophalangeal joints.

We conclude that Gran B is able to degrade proteoglycans but not collagen of articular cartilage. The presence of Gran B positive cells at the invasive pannus supports the view that this serine proteinase may contribute to joint destruction in RA.

INTRODUCTION

Proteolytic degradation of articular bone and cartilage is a characteristic feature of rheumatoid arthritis (RA). Cartilage destruction has mainly been attributed 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 (Gran), has been identified (2,3). Grans, which include Gran A, a proteinase with trypsin-like activity and Gran B, a proteinase which specifically hydrolyses after aspartic acid residues, are soluble cytolytic proteinases able to induce apoptosis in target cells in the presence of perforin (4,5). After release of the lysosome-like granules of activated cytotoxic lymphocytes and natural killer cells, Grans may also exert extracellular effects (6,7). Gran 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-a by monocytes (8,9). Furthermore, these enzymes may be involved in remodeling of extracellular matrix, as illustrated by the capacity of Gran A

to degrade basement membrane type IV collagen (10-14).
Several observations suggest a role of Grans in joint inflammation and destruction in patients with RA. In synovial fluid (SF) of patients with RA, lymphocytes have been shown to express Gran A messenger RNA (mRNA) (15,16). Recently, increased concentrations of soluble Gran B were found in SF of RA patients when compared with patients suffering from osteoarthritis or reactive arthritis (17). The levels of soluble Gran B were significantly higher in SF than in corresponding plasma samples indicating local production within the inflamed joint. These observations in SF are in line with those in rheumatoid synovial tissue (ST) where the presence of Gran A (18-20) and Gran B (19,20) has been reported. The number of Gran 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 acitivity (20).
To further investigate a potential role of Grans in the pathophysiology of joint destruction, we assessed the capacity of Gran B to degrade newly synthesized and resident proteoglycans (PGs) and collagen in bovine articular cartilage. In addition, we investigated the presence of Gran B containing cells at characteristic sites of cartilage destruction in RA being the chondro-synovial junction of metacarpophalangeal (MCP) joints.

MATERIALS AND METHODS

Production of radiolabeled cartilage matrix
In this study we used alginate beads to culture chondrocytes as described previously (21). This sytem provides a well characterized and convenient model to study the degradation of cartilage matrix. Chondrocytes cultured in alginate beads retain their cartilage-specific phenotype for at least 8 months. 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).
Chondrocytes were obtained from bovine metacarpophalangeal articular cartilage. Cartilage slices were digested in bacterial collagenase type 2 (Worthington Biochemical Corporation, New Jersey, 5mg/g cartilage, wet weight) at 37 °C overnight. After centrifugation and washing, chondrocytes were suspended in a low viscosity (1.2%) alginate gel at a density of $4x10^6$ cells/ml. Beads were formed by dropping the gel into a 102 mM $CaCl_2$ solution and incubated at room temperature for 20 minutes. The embedded chondrocytes were 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% carbon dioxide. After culturing for 4 days, 0.25 μCi/ml ^{35}S-sulphate and ^{3}H-proline

(Amersham International plc, Amersham, UK) were added to fresh medium to be incorporated into sulphated proteoglycans and collagen, respectively. Medium was changed twice weekly. After culturing for 28 days, the alginate gel was suspended in 55 mM sodium citrate to solubilize the beads and release the synthesized matrix. The solution was centrifuged (750 g, 6 minutes) and the matrix pellet was dissoved 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 with recombinant human Gran B (lot 10.5.95, 32 units/μl, specific activity 16.8 units/μg. Evanston Hospital Corporation, Evanston, IL) in phosphate buffered saline (PBS) with 0.05% (v/v) Tween-20 (PBST) (Janssen Chimica, Geel, Belgium) at 0.4 μg/ml and 0.04 μg/ml respectively, during 24 hours at 37 °C. Degradation 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 was counted by a liquid scintillation analyzer (Tri-Carb 1900 CA, Packard). Residual matrices were dissolved in 0.1 M NaOH. The percentage of matrix degradation was calculated as: (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 sacrifice of cows (1-2 years old). The specimens were thoroughly washed with 70% (v/v) ethanol and 2% Halamid-D (w/v) (AKZO, Wijk bij Duurstede, The Netherlands). Articular cartilage was removed aseptically from the joint, washed twice with PBS and prepared as slices with a wet weight of approximately 30 mg. 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 0.4 μg/ml and 0.04 μg/ml human Gran B ranging from 15 to 90 hours. Proteoglycan degradation was followed by measuring the release of sulphated 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 (Hyp) release into the culture medium by HPLC (23) (expressed as pmol/mg cartilage) and by measurement of *in situ* denatured collagen as described previously (24). Essentially, the latter method involves selective digestion of degraded collagen by α-chymotrypsin, hydrolysis in 6 M HCl of the released fragments as well as the residual tissue, followed by measurement of

the Hyp in both pools. The amount of denatured collagen in the cartilage is expressed as a percentage of the total amount of collagen: $100x(Hyp_{sup}/ [Hyp_{sup} + Hyp_{digested\ cartilage}])$.

Immunohistochemistry

Human metacarpophalangeal joints from 3 patients with rheumatoid arthritis were obtained at joint replacement. Slices of 3 mm were plastic-embedded in polymethacrylate (25). The tissue was fixed by overnight incubation in acetone at a constant temperature between -15 ^{0}C and -19 ^{0}C. Subsequently, the tissues were impregnated by constant rotation for 6 hours at 4 ^{0}C in molding cup trays (Polysciences, Warrington, PA). 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. Three-μm sections 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 Gran B (26) for 60 minutes. In 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 (27) 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 amino ethylcarbazole (Sigma, St. Louis, MO). Slides were counterstained with Mayer's Hämalaunlösung (Merck) and, after washing with distilled water, mounted in Kaiser's glycerol gelatin (Merck).The sections were washed between all steps with PBS and all incubations were carried out at room temperature.

Calculations and statistical analysis

All measurements 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 *Students t-test* for independent samples. All calculations were performed with the standard software package SPSS for MS Windows.

RESULTS

Incubation with Gran B results in degradation of newly synthesized proteoglycans of the chondrocyte extracellular matrix and leaves newly synthesized collagen unaffected.
Incubation with Gran B degraded the ^{35}S-sulphate labelled matrix after 24 hours. This effect was dose dependent as only 0.4 μg/ml Gran B resulted in a release of approximately 30% above the background level of ^{35}S from the matrix. Gran B in a concentration of 0.04 g/ml did not induce degradation of ^{35}S-sulphate labelled matrix (Figure 1A).
In contrast, Gran B did not induce the release of ^{3}H-pro labelled collagen after 24 hours of incubation at any of the concentrations tested (Figure 1B).

Gran B degrades resident proteoglycans and not collagen of cartilage explants.
Bovine articular cartilage explants were incubated with 0.04 and 0.4 μg/ml Gran B for 15 - 90 hours. Fifteen hours of incubation with 0.4 μg/ml Gran B resulted in a significant increase of GAG release up to 3.3 μg/mg cartilage. After 90 hours of incubation, both 0.04 μg/ml and 0.4 μg/ml Gran B increased GAG release to 6.1 and 9.2 μg/mg cartilage, respectively (Figure 2A)
No collagen degradation could be detected after 90 hours of incubation with both concentrations of Gran B; Hyp release did not rise above background levels (Figure 2B) and no change in the amount of denatured collagen was found in the explants (data not shown).

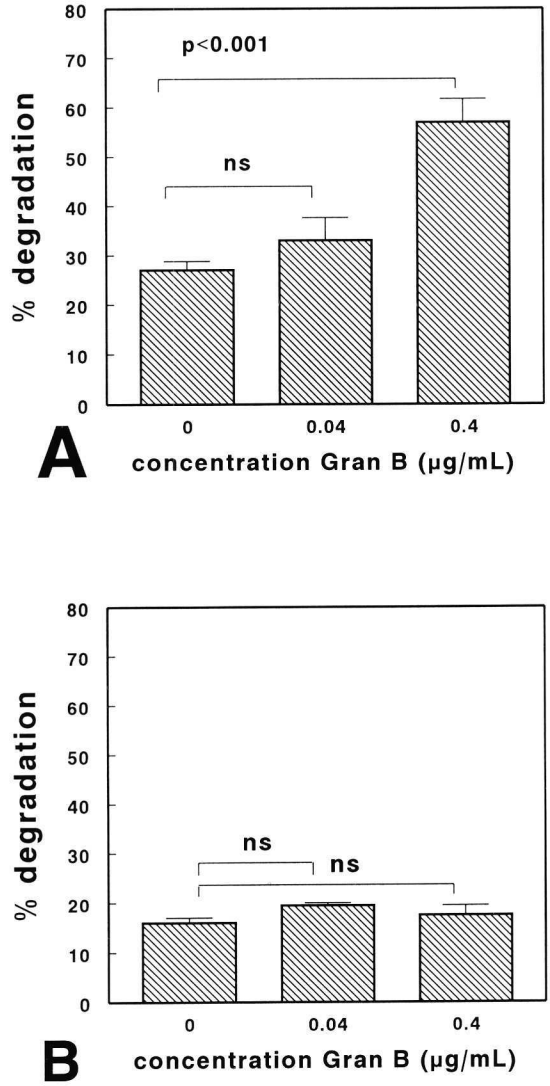

*Figure 1. Degradation of $^{35}SO_4$ labeled (panel A) and of ^{3}H-Pro labeled (panel B) cartilage matrix cultured in alginate beads for 4 weeks after incubation with 0.04 and 0.4 µg/ml human Granzyme B (Gran B) during 24 hours. Results are given as mean and standard error of the mean of radioactivity released in the culture medium as a percentage of the total radioactivity (n=3). *Statistically siginificant difference ($p < 0.05$; t-test for independent samples).*

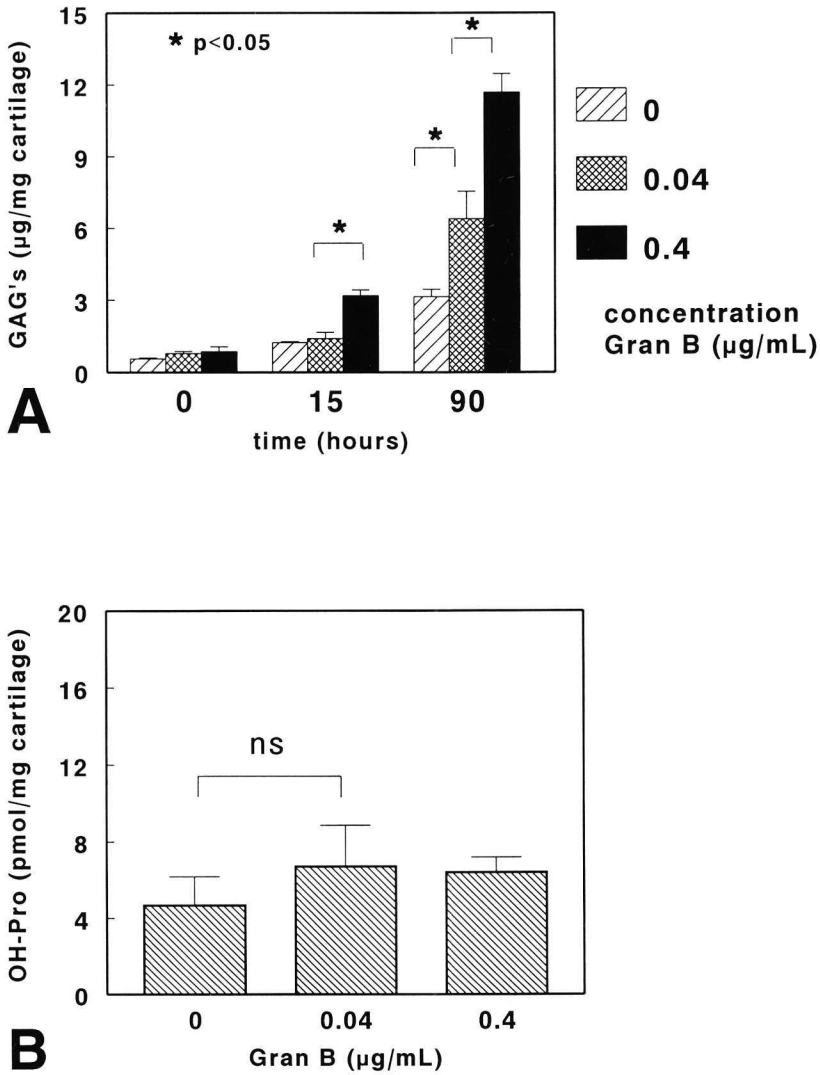

*Figure 2. Release of glycosaminoglycans (GAG; panel A) and hydroxy-proline (OH-Pro; panel B) from bovine articular cartilage explants after incubation with 0.04 and 0.4 µg/ml human Gran B during 15-90 hours. Results are given as the mean and standard error of the mean GAG (µg) or OH-Pro (pmol) released in the culture medium per mg cartilage (n=3). *Statistically siginificant difference ($p < 0.05$; t-test for independent samples).*

Localization of Granzyme B positive cells at the cartilage-pannus junction. Immunohistologic analysis of plastic-embedded sections of metacarpophalangeal joints from patients with rheumatoid arthritis yielded Gran B positive cells in synovial tissue at the invasive pannus (Figure 4). These cells showed a granular pattern and were found in close association with the articular cartilage and bone. Staining was negative when the primary antibody was omitted or irrelevant antibodies were applied as controls.

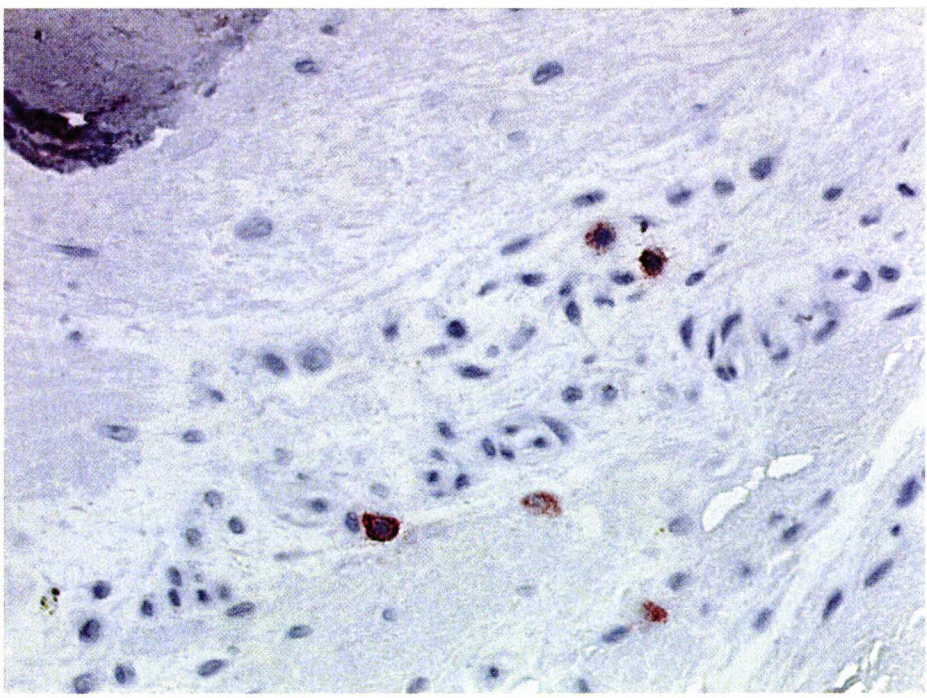

Figure 3. Localization of Gran B positive cells in invasive pannus in metacarpophalangeal joints of patients with rheumatoid arthritis. (Single-stain peroxidase technique; Mayer's Hämalaunlösung counterstained; original magnification x 250)

DISCUSSION

RA is a progressive, destructive disease affecting MCP and metatarsophalangeal joints most frequently. Within the joint, the inflamed hyperplastic synovial 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 (28). 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 held responsible for tissue destruction in RA. Much emphasis has been placed on serine proteinases and matrix metalloproteinases (9). Recently, Grans have also been suggested to play a role in the degradation of extracellular matrix (10-13).
In the present study, we further addressed the question whether Gran B is involved in cartilage degradation of RA patients. The capacity of Gran B to degrade articular cartilage matrix was assessed *in vitro,* and *in vivo* expression of Gran B containing cells was investigated in synovial tisue at the pannus-cartilage-bone junction of MCP joints from RA patients.
In a previous study, the PG degrading activity of IL-2 stimulated human lymphocyte cell lysates was attributed to Gran B (11). The proteolytic activity of these total cell lysates, putatively containing Gran B, became apparent by the capacity to release ^{35}S-labeled matrix components from chondrocyte monolayers and by lowering the molecular weight of ^{35}S-aggrecan in overnight-radiolabeled cartilage explants. ^{35}S pulse-labeling provided the opporunity to follow degradation of newly synthesized (non-resident) proteoglycans. The objective of our study was to explore whether Gran B is also able to degrade more resident tissue components to provide further support for a cartilage destructive role of Gran B under pathophysiological conditions. The availability of purified human Gran B provided the opportunity to directly investigate the capacity of this enzyme to degrade an organized cartilage matrix, produced during 4 weeks of chondrocyte culture (22), and to degrade explants of intact bovine articular cartilage.
Incubation with Gran B resulted in degradation of PGs incorporated in a three-dimensional network of collagen produced by articular chondrocytes. In contrast to PG degradation, no significant release of ^{3}H-Pro could be detected, indicating lack of collagenolytic activity of Gran B in this system. The present data reveal that Gran B also mediates degradation of resident PGs. The capacity of human Gran B to release GAGs from whole articular cartilage explants indicates extensive digestion of PGs to protein fragments which are small enough to diffuse out the tissue. The PG degrading activity of Gran B is illustrated by the catalytic efficiency of Gran B for digestion of aggrecan which is approximately 400-fold greater than the catalytic efficiency of for instance human stromelysin-1 (11). Unlike the capacity of Gran A to preferentially degrade the α2 chain of basement membrane type IV collagen (13), Gran B was not found to degrade the collagen component of articular cartilage explants.

Previous studies have clearly shown that the expression of Gran B is specifically increased in the rheumatoid joint. Levels of soluble Gran B in synovial fluid were strongly elevated in patients with RA as compared with osteoarthritis or reactive arthritis affected patients (17). In synovial tissue, specifically increased expression at the level of both mRNA for Gran B and Gran B protein was found (19,20). These Gran B positive cells, mainly natural killer cells, were seen in the synovial lining layer and in the sublining area, but the presence of these cells at the particular site where erosions develop was never investigated. The demonstration of Gran B containing cells at this specific site requires the preservation of the chondro-synovial 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 Gran B. We here demonstrate that Gran B positive cells can be present at the pannus invasive front.

The previously demonstrated increased numbers and the location of these Gran B positive cells suggests that cytotoxic lymphocytes can be involved in matrix degradation in several ways: by cell-cell interactions, by cytokine-mediated stimulation of matrix degrading synoviocytes, as well as by local production of matrix degrading enzymes, including Gran B. It can be envisaged that production of Gran B in close proximity to cartilage, active binding of positively charged Gran B to the anionic extracellular matrix and the apparent absence of an endogenous inhibitor may lead to local concentrations effective to degrade proteoglycans.

The results of the present study serve to contribute that human Gran B is capable of degrading the proteoglycan component of cartilage and can be produced in close proximity to the invasive pannus. These findings, together with the previously observed increased expression of Gran B in rheumatoid synovial joints suggest a substantial role of Gran B in the continuous destruction of cartilage in RA.

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CHAPTER 7

SUMMARY AND DISCUSSION

In this chapter, the results of the studies on proteolysis in rheumatoid arthritis (RA) will be summarized and discussed.
This thesis focusses on the the possible involvement of the plasminogen activation (PA) system and granzyme B in rheumatoid joint destruction.
In **Chapter 1**, a general introduction to clinical features, and concepts of the pathophysiology of RA are given. The major constituents of connective tissue, being collagen and proteoglycans, are described in more detail. Degradation of connective tissue, including bone and cartilage, is a characteristic feature of RA. This joint destruction depends largely upon the excessive production of proteolytic enzymes by the hyperplastic, inflamed synovial tissue. The PA system, able to degrade extracellular matrix proteins directly and indirectly by activation of latent matrix metalloproteinases, may be one of the mechanisms by which rheumatoid synoviocytes destroy their pericellular environment. Clinical and experimental evidence, reported previously by other investigators, suggests a contribution of the PA system to bone and cartilage destruction in RA. Allmost all cells present in the joint have been shown to produce plasminogen activators, and their inhibitors (PAI). The specifically increased expression of components of the PA system in the rheumatoid joint has been associated with parameters of arthritis activity and joint destruction.
To find further evidence for a possible pathophysiological role of the PA system in RA, we quantified antigenic levels of urokinase type-PA (u-PA), tissue type-PA (t-PA), PA inhibitors (PAI-1, PAI-2) and u-PA receptor (u-PAR) in extracts of synovial tissue from patients suffering from RA and osteoarthritis, and studied the distribution and staining intensity of these components in synovial tissue by immunohistochemistry.
As described in **Chapter 2**, RA synovial tissue homogenates contained variable but significantly higher concentrations of u-PA, PAI-1, PAI-2 and u-PAR than synovial tissue from osteoarthritis patients. Immunohistochemically, u-PA, u-PAR and PAI were mainly located in the synovial lining area, which is generally considered to be the "invasive front" of inflamed synovial tissue. These findings fit within the concept of localized, u-PA mediated degradation of articular structures. In contrast to osteoarthritic synovium, concentration of t-PA was decreased, and by immunohistochemical staining it was hardly detectable in sections of RA synovial tissue. These results are consistent with the previously reported local increase of u-PA at the expense of t-PA in rheumatoid SF, and have been confirmed by others at the mRNA level in the mean time. The altered ratio of t-PA to u-PA may reflect a shift from effective fibrinolysis, leading to intra-articular fibrin accumulation, towards extracellular matrix proteolysis, as reflected by joint destruction.
Although these observations support a role of the PA system in RA, detailed knowledge of the mechanisms and components involved is lacking, and little information exists on its possible capacity to degrade specialized matrices like bone and cartilage.

Chapter 3 concerns the potential of the PA system to degrade bone matrix *in vitro*. Degradation of non-mineralized and mineralized bone matrix was achieved by mouse cells producing human u-PA in the presence of plasminogen, but not by cells expressing u-PAR. Co-cultivation of u-PA and u-PAR expressing cells resulted in a marked increase of degradation, most pronounced at low levels of u-PA. Inhibition by anti u-PA antibodies, tranexamic acid (an inhibitor of plasminogen activation), aprotinin (a plasmin inhibitor) and by interference with the u-PA/u-PAR interaction, indicate the functional involvement of u-PA mediated plasminogen activation, particularly when present at the cell surface. Our results are in accordance with PA mediated bone matrix degradation by osteosarcoma cells reported by others, and make this enzyme system a potential contributor to RA-associated bone destruction.

Besides bone degradation, plasminogen activation may also contribute to rheumatoid cartilage degradation. **Chapter 4** describes the capacity of isolated RA synovial fibroblasts, by activating plasminogen, to degrade cartilage matrix, as well as explants of intact articular cartilage. Plasminogen activation was achieved by u-PA, which was produced by the rheumatoid synoviocytes.

We found that degradation of cartilage matrix could be reduced by either an exogenous plasmin antagonist or expression of a plasmin inhibitor at the surface of degrading cells by gene transfer. Infection of rheumatoid fibroblast-like synoviocytes with an adenovirus encoding a plasmin inhibitor, which binds u-PAR, could affect matrix degradation in two ways. Firstly, by reduction of pericellular proteolysis through inhibition of plasmin. Secondly, by competitively disturbing the u-PA/ u-PAR interaction, leading to reduction of matrix degradation as we described in chapter 4.

The release of collagen degradation products from the matrix and tissue indicated collagenolytic activity. Since plasmin has only moderate capacity to degrade components of the collagen molecule, activation of latent matrix metalloproteinases is probably involved in the cartilage degradation.

Intervention with the PA system *in vivo* provides the opportunity to learn more about its relative importance in proteolytic joint destruction and may even offer perspectives for the development of joint protective therapy.

In **Chapter 5** we present the results of a study in which we evaluated the effect of tranexamic acid (TEA), an inhibitor of plasminogen activation, on urinary excretion of collagen degradation products in an animal model of arthritis (rat adjuvant arthritis) and in patients suffering from RA. In the adjuvant model of arthritis, TEA reduced excretion rates of collagen crosslinks towards control level. In an open label pilot study with RA patients, TEA decreased the excretion rates after 8 weeks of treatment, whereas this suppressive effect disappeared when TEA administration was discontinued. The exact mechanism underlying these effects is unknown. It can be envisaged that competitive interference of TEA with plasminogen for binding sites on the cell surface and matrix, leads to inhibition of net plasmin activity.

The main finding of this study is that administration of an anti-proteolytic agent, aimed at the level of matrix degrading proteinases in arthritis, can result in a decrease of collagen degradation. This effect may prove to be advantageous to patients with RA in the future.

Granzymes, a set of serine proteinases being produced by cytotoxic cells and specifically upregulated in the rheumatoid joint, may also possess matrix degrading properties involved in the development of joint damage. In **Chapter 6** we addressed the question whether granzyme B might contribute to degradation of articular cartilage. granzyme B, best known for its capacity to induce apoptosis in target cells in the presence of perforin, is produced by activated cytotoxic lymphocytes and natural killer cells, and its expression is specifically increased in synovial fluid and tissue of RA patients.
We assessed the capacity of this enzyme to degrade articular cartilage *in vitro* and studied its presence at the characteristic site of cartilage destruction, being the chondro-synovial junction of rheumatoid metacarpophalangeal joints. The results of this study serve to contribute that human granzyme B is capable of degrading the proteoglycan component of cartilage and can be produced in close proximity to the invasive pannus. Our findings, together with the previously reported increased expression in RA joints suggest that cytoxic lymphocytes can be involved in the continuous destruction of cartilage in RA in several ways; by cell-cell interactions, by cytokine mediated stimulation of matrix degrading fibroblasts or macrophages, as well as by local production of matrix degrading enzymes, including granzyme B, at the site where cartilage is eroded.

CONCLUSION AND PERSPECTIVES

Synovial tissue of patients with RA were found to contain considerably increased amounts of u-PA, u-PAR and PAI, compared with patients suffering from osteoarthritis. These observations are in line with previous findings in rheumatoid synovial fluid and suggest a pathophysiological role of the PA system in inflammatory joint destruction. Further evidence was obtained from *in vitro* studies in which we observed the capacity of u-PA producing cells to degrade bone and cartilage matrix in the presence of plasminogen. Fibroblasts, expressing u-PA, degraded bone-like matrix, an effect that could be stimulated by interaction with u-PAR producing cells. Human rheumatoid synovial fibroblasts produced u-PA and were found to degrade cartilage matrix in a plasmin dependent way. Degradation could be inhibited by an exogenous as well as an endogenous plasmin inhibitor, the latter being expressed by the fibroblasts through gene transfer. Interference with the PA system *in vivo* also indicated involvement of this enzyme system in rheumatoid tissue degradation. Tranexamic acid, an inhibitor of plasminogen activation, reduced the excretion of collagen degradation products both in an experimental model for arthritis and in patients with RA.
Granzyme B, demonstrated in human rheumatoid synovial tissue at the chondro-synovial junction, was found to degrade proteoglycans of articular cartilage.

Despite increasing understanding of the pathogenesis of RA and the development of various therapeutic strategies, rheumatologists remain powerless to inhibit the progress of joint destruction. To change the situation, at least two problems need to be resolved. Firstly, more insight in cells and mechanisms involved in this destructive process needs to be obtained to find possible targets for joint protective intervention. Secondly, new methods to assess a possible joint protective effect should be developed. The gold standard, radiological progression, requires follow-up studies over time spans of at least 2 years, and large numbers of patients. There is great need for biochemical markers which provide a suitable surrogate, able to predict or reflect the process of joint destruction.
Increasing knowledge of the PA system addresses to both points. The increased expression of the PA system in the rheumatoid joint, and its capacity to degrade both bone and cartilage matrix, suggest a pathophysiological role. The exact mechanism of PA mediated bone and cartilage degradation needs to be determined. Plasmin has been shown to degrade most extracellular proteins directly. A previously suggested proteolytic cascade including the PA system and matrix metalloproteinases is also supported from the results of this study and merits further exploration.
The findings of this study make this enzyme system a potential candidate for therapeutic intervention. Future research will learn whether interference with the PA system in patients with RA results in clinically significant joint protection.

Although tranexamic acid is probably not the most potent anti-proteolytic drug, its availability and mild toxicity profile makes it a suitable lead compound to investigate the therapeutic principle of antirheumatic intervention at the level of matrix degrading proteinases. A placebo-controlled trial should be the next step to confirm its effect on collagen crosslink excretion and radiological joint damage in patients suffering from RA.
Gene transfer, aimed to reduce proteolytic activity of bone and cartilage degrading synoviocytes may be another potential way to reduce joint damage in RA. In this respect, the observed inhibition of cartilage matrix degradation by transfection of RA synoviocytes with a virus coding for an u-PAR binding plasmin antagonist is promising. This technique offers great opportunities to study the effect of genes encoding (combinations of) proteinase inhibitors and receptor binding proteins on joint destruction.
Analogous to the prognostic significance of u-PA and PAI-1 in malignant tumors, the increased expression of u-PA, u-PAR and PAI in synovial fluid and /or synovial tissue may have a prospective role in predicting or distinguishing rapidly progressive- from slowly progressive erosive RA. Both cross-sectional and longitudinal studies will be necessary to validate PA components in this respect.
The expression of granzymes in RA synovial fluid and/or tissue as a possible biological marker to predict or monitor the development of joint damage is currently under investigation.

The results of this thesis provide the rationale for further research to explore possible application of the PA system and granzyme B as prognostic markers and as targets for therapeutic intervention.

SAMENVATTING

Reumatoïde arthritis (RA) is een chronisch invaliderende gewrichtsziekte die vaak gepaard gaat met uitval uit het arbeidsproces, ernstige aantasting van de kwaliteit van het leven en toenemende zorgbehoefte. In westerse landen komt de ziekte bij circa 1% van de bevolking voor. Kenmerkend is de chronische ontsteking van gewrichten die leidt tot pijnlijke stijfheid, zwelling en vaak ook onherstelbare beschadiging met functieverlies. Naast de gewrichten kunnen ook andere organen zijn aangedaan, zoals huid, longen, ogen, hart en bloedvaten.
Reumatoïde artritis is het gevolg van activiteit van het afweersysteem. De oorzaak van de schadelijke activiteit van het afweersysteem voor het lichaam, is onbekend. Volgens de huidige inzichten moet deze worden gezocht in een combinatie van omgevingsfactoren (zoals bijvoorbeeld infecties) en genetische aanleg.
Ook hormonale factoren lijken een rol te spelen. Reuma komt drie maal vaker bij vrouwen voor en verbetert vaak tijdens de zwangerschap.
De tot op heden beschikbare behandelmethoden zijn beperkt, hebben veel negatieve bijwerkingen en richten zich vooral op het verminderen van de ontstekingsactiviteit. Hoewel gewrichtspijn, -stijfheid en -zwelling met wisselend succes kunnen worden bestreden blijkt echter het voortschrijden van de invaliderende schade aan de gewrichten nauwelijks te kunnen worden beinvloed. De laatste jaren groeit het inzicht dat gewrichtsontsteking en gewrichtsdestructie niet onlosmakelijk met elkaar verbonden zijn, maar twee processen zijn die in het beloop van de ziekte een onafhankelijk beloop nemen. Ontstekingsremmende therapie, hoe succesvol deze ook moge zijn, vermindert de schade aan het gewricht onvoldoende.
Inmiddels bestaan er veel aanwijzingen dat weefselafbrekende enzymen (proteolytische enzymen), afkomstig uit het ontstoken gewrichtskapsel, betrokken zijn bij de afbraak van bot, kraakbeen en omliggende gewrichtsstructuren.
Serine proteinasen zoals de enzymen van het Plasminogeen-Activatie (PA) systeem en granzymen, en Matrix-Metalloproteinasen (MMPs), lijken elk afzonderlijk en in hun onderling funtioneel verband een belangrijke rol te vervullen. Interventie gericht op het beperken van de activiteit van deze enzymen zou een eerste stap kunnen zijn tot het remmen van gewrichtsschade.
Eén van de enzymsystemen die een belangrijke rol wordt toegedacht bij het ontstaan van gewrichtsschade is het het PA systeem. Plasminogeen activering geschiedt onder invloed van plasminogeen activatoren en leidt tot de vorming van het actieve enzym plasmine. Dit enzym is niet alleen betrokken bij het oplossen van fibrine en bloedstolsels (fibrinolyse en trombolyse), maar lijkt ook een belangrijke functie te vervullen in de buiten de cel gelegen eiwitafbraak die nodig is voor weefselombouw en celmigratie. De plasmine gemedieerde, extracellulaire weefselombouw vormt een essentieel onderdeel van diverse fysiologische en pathologische processen zoals wondherstel, angiogenese, tumorinvasief gedrag en ontsteking.

Uit eerder onderzoek is gebleken dat bij patiënten met reumatoïde artritis, het gewrichtsvocht een verhoogde concentratie van componenten van het PA systeem bevat. Mogelijk dat dit enzymsysteem betrokken is bij het ontstaan en onderhouden van gewrichtsschade bij patiënten met reumatoïde artritis.
Matrix metalloproteinasen zijn krachtige weefsel afbrekende enzymen die een belangrijke uitvoerende rol bij (patho-)fysiologische weefselombouw vervullen. Het MMP-proteolytisch potentieel staat onder strikte controle. Zo wordt de produktie van latente (pro-) MMPs gereguleerd door immunologische mediatoren en vindt activering plaats door de werking van andere enzymen, waaronder plasmine. Tevens bestaat er een uitgebreid systeem van natuurlijke remmers om de uiteindelijke netto activiteit te bewaken.
Verhoogde concentratie en activiteit van MMPs zijn gevonden in zowel het gewrichtsvocht als het perifere bloed van patiënten met reumatoïde artritis. Hun betekenis bij gewrichtsdestructie is onomstreden.
Ook tumoren blijken componenten van het PA systeem en MMPs te bevatten. Eerdere studies hebben een verband aangetoond tussen de mate van expressie van deze enzymen en het vermogen van de tumor om in het omliggend weefsel in te groeien en uit te zaaien.
De gelijkenis tussen tumorweefsel en ontstoken gewrichtskapsel (synovium) dringt zich op. Het sterk verdikte reumatoïde synovium overwoekert en invadeert bot, kraakbeen en omliggende structuren. De overeenkomst tussen het invasieve gedrag van tumorcellen en reumatoïde synoviumcellen nodigt uit tot verdere studie naar het verband tussen bovengenoemde enzymsystemen en reumatoïde gewrichtsdestructie.
Granzymen, waaronder granzyme A en B zijn recent ontdekte serine proteinasen die door een specifieke groep ontstekingscellen (cytotoxische T cellen en natural killer cellen) worden geproduceerd en van belang zijn bij het doden van cellen. Granzyme B wordt in hoge concentraties aangetroffen in het gewrichtskapsel van patiënten met reumatoïde artritis.
Over de mogelijke rol van het PA systeem en granzymen bij reumatoïde gewrichtsdestructie is nog weinig bekend. In dit proefschrift wordt onderzoek beschreven dat gericht is op het verkrijgen van meer inzicht in de functionele betekenis van het PA systeem en granzymen in het ontstaan van bot- en kraakbeenschade. Tevens is gezocht naar aangrijpingspunten voor de ontwikkeling van gewrichtsbeschermende therapie.
Hoofdstuk 1 geeft een overzicht van de belangrijkste bouwstenen van bot en kraakbeen, en beschrijft het PA systeem, MMPs en granzymen. Verder wordt de mogelijke rol van deze enzymen bij reumatoïde artritis behandeld.
Hoofdstuk 2 beschrijft het voorkomen en de lokalisatie van de componenten van het PA systeem in het synovium van patiënten met reumatoïde artritis, vergeleken met patiënten die aan artrose leiden. Het blijkt dat bijna alle componenten (u-PA, PAI-1, PAI-2, u-PAR), met uitzondering van tissue-type plasminogeen activator (t-PA), in verhoogde mate voorkomen in de verdikte binnenbekleding (synovial lining) van het synovium van RA patienten.

De resultaten zijn in overeenstemming met eerdere bevindingen in het synoviale vocht en wijzen op een verhoogd activiteitsniveau van dit enzymsysteem in het chronisch ontstoken gewricht. Mogelijk dat onderdrukking van t-PA, dat een belangrijke rol speelt bij het oplossen van fibrine, bijdraagt aan ophoping van fibrine in het ontstoken gewricht.
Een verhoogd vóórkomen van het PA systeem zegt nog niet zoveel over een eventuele rol bij gewrichtsafbraak. In **hoofdstuk 3** worden de resultaten gepresenteerd van een studie naar het mogelijke botmatrix afbrekend vermogen van het PA systeem. Twee typen muis-fibroblasten, die humaan urokinase-type plasminogeen activator (u-PA) of u-PA receptor (u-PAR) tot expressie brengen werden gekweekt op een laagje botmatrix in de aanwezigheid van plasminogeen.
U-PA producerende cellen veroorzaakten duidelijk botmatrixafbraak. Deze afbraak kon o.a. worden geremd met de plasmineremmer aprotinine en tranexaminezuur. Dit laatste middel remt plasminogeen activering en wordt klinisch toegepast als anti-fibrinolyticum (cyklokapron®). De fibroblasten die alleen u-PAR tot expressie brachten waren daarentegen niet in staat tot degradatie. Het kweken van relatief kleine hoevelheden u-PA producerende cellen in de aanwezigheid van u-PAR producerende cellen leidde tot een stimulering van het botmatrix degraderend vermogen. Hiertoe bleek binding tussen u-PA en zijn celgebonden receptor noodzakelijk.
De bevinding dat het PA systeem in staat is tot afbraak van botmatrix *in vitro*, ondersteunt een mogelijk belang bij gewrichtsafbraak in RA.
Dat ook reumatoïde synoviale fibroblasten in staat zijn tot plasminogeen activering die kan resulteren in weefselschade, wordt beschreven in **hoofdstuk 4.** Deze cellen werden geïsoleerd uit het ontstoken gewrichtskapsel van RA patiënten, die een operatie moesten ondergaan. Vervolgens werden de cellen gekweekt op een laagje kraakbeenmatrix en explantaten van runderkraakbeen. De fibroblasten bleken in staat tot produktie van u-PA en brachten u-PAR tot expressie. Toevoeging van plasminogeen aan het kweeksysteem resulteerde in afbraak van de kraakbeenmatrix en het runderkraakbeen. De plasmine remmer aprotinine bleek ook hier in staat de kraakbeenafbraak te voorkomen. Omdat aprotinine een smalle therapeutische breedte heeft, is het middel niet geschikt is voor eventuele systemische toediening bij RA patiënten. Lokale toepassing of produktie binnen het gewricht van deze plasmineremmer, zou de voorkeur genieten. Met behulp van moleculair biologische technieken bleek het mogelijk om de humane reumatoïde fibroblasten een gemodificeerde vorm van aprotinine te laten produceren. Hiertoe werden de cellen geïnfecteerd met een virus dat codeert voor aprotinine, dat specifiek bindt aan u-PAR. De zo veranderde synoviale cellen bleken niet meer in staat tot kraakbeenafbraak. De resultaten van deze studie openen wellicht de mogelijkheid tot de ontwikkeling van gentherapie, die zich richt op lokale bescherming van het kraakbeen in het ontstoken gewricht.
De experimenten zoals beschreven in hoofdstuk 3 en 4 zijn uitgevoerd *in vitro*. De vraag werpt zich op of toediening van een middel, dat zich richt op remming van het

PA systeem, ook *in vivo* effect zou hebben. In **hoofdstuk 5** worden de resultaten beschreven van een studie naar het effect van tranexaminezuur bij proefdieren met artritis en patiënten met reumatoïde artritis.
In het proefdiermodel, werd in de vroege fase van de gewrichtsontsteking en beschermend effect op de afbraak van collageen waargenomen bij de met tranexaminezuur behandelde dieren.
Op het moment dat de zeer destructieve artritis zich volledig had ontwikkeld kon dit effect niet meer worden waargenomen. Ook röntgenfoto's, gemaakt aan het einde van de studie toonden geen effect van de behandeling.
In een pilot studie, met een duur van zestien weken, werden negen patiënten met RA gedurende 3 maanden behandeld met tranexaminezuur. Na acht en twaalf weken behandeling was er een significante daling in de uitscheiding van collageen afbraakprodukten waarneembaar.
Vier weken na het einde van de behandeling bleek deze daling in uitscheiding volledig teniet gedaan en bevond de excretie van collagen afbraakprodukten zich weer op uitgangsniveau.
Tranexaminezuur heeft weliswaar een bescheiden effect op de afbraak van collageen bij artritis, maar de bevindingen suggeren een rol van het PA systeem bij de gewrichtsdestructie. Voor zover ons bekend, is dit de eerste observatie van het gunstig effect van een middel dat zich primair richt tegen de proteolytische, extracellulaire matrixafbraak bij patiënten met reumatoïde artritis.
Hoofdstuk 6 beschrijft het vermogen van het recent ontdekte granzyme B om proteoglycanen in kraakbeen en kraakbeenmatrix af te breken. Granzyme B bleek echter niet in staat tot afbraak van het collageen in kraakbeen. Met behulp van een speciale immunohistochemische techniek bleek het mogelijk om granzyme B positieve cellen aan te tonen in het reumatoïde gewrichtskapsel, op de specifieke plaats waar het synovium overgrijpt op het gewrichtskraakbeen. Deze bevindingen, samen met de in eerdere studies verhoogde expressie van granzymen in gewrichten van patiënten met reumatoïde artritis, ondersteunen een pathofysiologische betekenis van deze serine proteinasen bij het ontstaan en onderhouden van kraakbeenschade.

Ondanks toenemend begrip van de pathofysiologie en de ontwikkeling van nieuwe behandelingsmethoden blijken reumatologen onvoldoende in staat om het voortschrijden van gewrichtsschade bij reumatoïde artritis te beperken. Om hier verandering in te brengen, moet meer kennis worden vergaard over de mechanismen van destructie en eventuele aangrijpingspunten voor specifieke gewrichtsbeschermende therapie. Tevens moeten nieuwe methoden worden ontwikkeld om het proces van gewrichtsschade en het effect van eventuele interventie te vervolgen. De huidige gouden standaard, radiologische progressie, vergt een langdurig vervolgen van grote aantallen patiënten en bemoeilijkt effectief onderzoek.

Mogelijk dat in de toekomst biochemische markers voor bot- en kraakbeenmetabolisme een geschikte maat kunnen vormen voor het voorspellen of weerspiegelen van gewrichtsdestructie.
Parallel aan de prognostische betekenis van u-PA en plasminogen activator inhibitor-1 (PAI-1) bij tumoren zou de toegenomen expressie van u-PA, u-PAR, PAI-1 in het synoviale vocht en -weefsel mogelijk ook voorspellende betekenis kunnen hebben in het onderscheid tussen snelle en langzame progressie van de schade bij reumatoïde artritis. Prospectieve studies zullen de waarde van deze parameters moeten aantonen. Momenteel vindt onderzoek plaats naar de waarde van de concentratie van granzyme B in synoviaal weefsel en -vocht als voorspeller van klinisch beloop.
De bevindingen in dit proefschrift leveren nieuwe gegevens op over de pathofysiologische betekenis van het PA systeem. De toegenomen expressie van het PA systeem in reumatoïde gewrichten en het vermogen bot- en kraakbeenmatrix af te breken suggereren een rol bij het ontstaan van gewrichtsschade. De eerder veronderstelde proteolytische cascade van het PA systeem en MMPs zou hierbij van groot belang kunnen zijn, en vormt een vruchtbaar onderzoeksveld. De toekomst zal leren of interventie, die zich specifiek richt op remming van het PA systeem, een klinisch relevant gewrichtssparend effect zal hebben. Hoewel misschien niet het meest krachtige middel, lijkt tranexamine zuur, gezien de beschikbaarheid en veiligheid, geschikt om het therapeutisch principe van anti-reumatische interventie op het niveau van weefselafbrekende enzymen verder te onderzoeken. Een placebo-gecontrolleerde studie is de volgende stap om het effect op weefselschade te onderzoeken.
Gentherapie, gericht op remming van afbraak van bot- en kraakbeen door synoviale cellen vormt een andere mogelijkheid om gewrichtsdestructie te remmen. De in dit proefschrift beschreven remming van kraakbeenafbraak door synoviumcellen van RA patiënten, die getransfecteerd zijn met gemodificeerd, celbindend aprotinine is in dit verband veelbelovend. Deze techniek. biedt de mogelijkheden om ook andere enzymremmers in combinatie met elkaar, of met receptorbindende eiwitten te onderzoeken op hun matrixbeschermend vermogen.
Concluderend kan worden gesteld dat de resultaten van dit proefschrift een pathofysiologsiche betekenis van het het PA systeem en granzyme B bij gewrichtsschade van reuma patiënten, ondersteunen. De bevindingen bieden houvast voor het verder verkennen van mogelijkheden om het PA systeem en granzyme B als prognostische markers en als aangrijpingspunt voor therapeutische interventie toe te passen.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 19 augustus 1960 te Monster.
In 1978 werd het diploma Voorbereidend Wetenschappelijk Onderwijs behaald aan het Groen van Prinsterer College te Den Haag. In dat zelfde jaar begon hij met de studie Fysiotherapie aan de Opleiding voor Fysiotherapie te Leiden. In 1979 begon hij met de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam, aan welke hij in 1987 het artsexamen behaalde. Tijdens de studie Geneeskunde was hij van 1981 tot en met 1984 als student-assistent verbonden aan de vakgroep Neuroanatomie, en van 1986 tot en met 1987 aan de afdeling Neuroradiologie. In 1987 en 1988 werd de militaire dienstplicht vervuld bij het Selectie Centrum Koninlijke Landmacht voor beroepspersoneel te Hilversum.
In november 1988 werd hij als arts-assistent op de afdeling Interne Geneeskunde van het Ziekenhuis Leyenburg te Den Haag aangesteld. Vanaf 1990 tot 1993 januari volgde hij daar de opleiding Interne Geneeskunde (opleider: Dr. J.C.M. van der Vijver).
Vervolgens werd de opleiding tot reumatoloog voortgezet in het Academisch Ziekenhuis te Leiden (opleider: Prof. dr. F.C. Breedveld).
Sinds januari 1993 is de schrijver tevens verbonden aan het Gaubius Laboratorium, TNO Preventie en Gezondheid, te Leiden. Aldaar werd op de afdeling Vaat- en Bindweefsel Onderzoek, onder begeleiding van Dr. J.H. Verheijen, en in samenwerking met de afdeling Reumatologie van het Academisch Ziekenhuis Leiden het onderzoek uitgevoerd dat wordt beschreven in dit proefschrift.
In januari 1996 werd de schrijver ingeschreven in het specialistenregister als reumatoloog, waarna hij verbonden bleef aan de afdeling Reumatologie als chef de clinique.
Sinds oktober 1996 is hij werkzaam als reumatoloog in het Ziekenhuis Leyenburg, te Den Haag.

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