Review

The urokinase-type plasminogen activator system and inflammatory joint diseases

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ABSTRACT.
Much evidence indicates that the urokinase plasminogen activator (u-PA), the urokinase receptor (u-PAR) and the serpin inhibitors are critical in cell invasion processes. The balance between u-PAR-bound u-PA and inhibitors modulate a pericellular proteolytic activity able to give "stop and go" signals to invading cells. The plasminogen activation system operates both directly and in concert with the matrix-metalloproteinase system. Direct interactions of u-PAR with vitronectin and integrins further regulate cell invasion. Another line of evidence suggests that u-PA-u-PAR interaction elicits chemotaxis, chemoinvasion and cell multiplication, events that do not require plasmin generation and therefore are referred to as "plasminogen-independent".
Following the description of the main molecular and functional characteristics of the cell-surface-associated plasminogen activation system, we discuss here the observations indicating a role of this system in many aspects of the rheumatic diseases, ranging from the infiltration of inflammatory cells into the affected joint, infiltration of synovial cells into the underlying cartilage, and remodeling of the cartilage itself. Evidence of the intra-articular cytokine- and growth factor-dependent regulation of the components of the plasminogen activation system are presented in terms of the paracrine and autocrine regulation of receptor-associated fibrinolysis. The roles of plasminogen-dependent and plasminogen-independent u-PAR-associated events in various phases of joint inflammation are also discussed. A knowledge of these processes is required for the therapeutic utilization of antagonists of the u-PA/u-PAR system able to control the activity of proliferating and invading cells in inflammatory joint diseases.

Introduction

Over the last decade much evidence has been provided, leading to a clear distinction between the functions of tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), which is now believed to be based on the presence of specific receptors for t-PA along the fibrin strands and of u-PA on the cell surface (u-PAR). Thus, while t-PA has been identified as the main plasminogen activator (PA) involved in thrombolysis *in vivo*, u-PA and its cellular receptor are critical in the cell-driven degradation of the extracellular matrix (ECM), which is at the basis of cell invasion within the surrounding tissues (for reviews see 1, 2, 3).

The term “invasion” connotes the ability of cells to cross the anatomical barriers separating tissue compartments (basement membranes, ECM, cell junctions). Invasion is a distinctive feature of the synovial pannus in rheumatoid arthritis (RA) (4), where many types of cell invasion occur: a) the invasion of new vessels into the proliferating synovial lining (5); b) erosion and invasion of the underlying articular cartilage by the synovial pannus itself; c) extravasation into the articular cavity of leukocytes, which releases many cytokines and inflammation factors. Cell-driven extracellular matrix destruction also takes place in osteoarthritis (OA), where cartilage remodelling is supported by perilacunar tissue degradation which closely resembles that associated with cell invasion (6).

Extracellular proteolytic enzymes (serine proteinases and metalloproteinases), have been implicated in cell invasion (3), the basic notion being that enzyme release facilitates cell invasion within degraded ECM and basement membranes. The possibility that plasminogen activation may play a pivotal role in this proc-

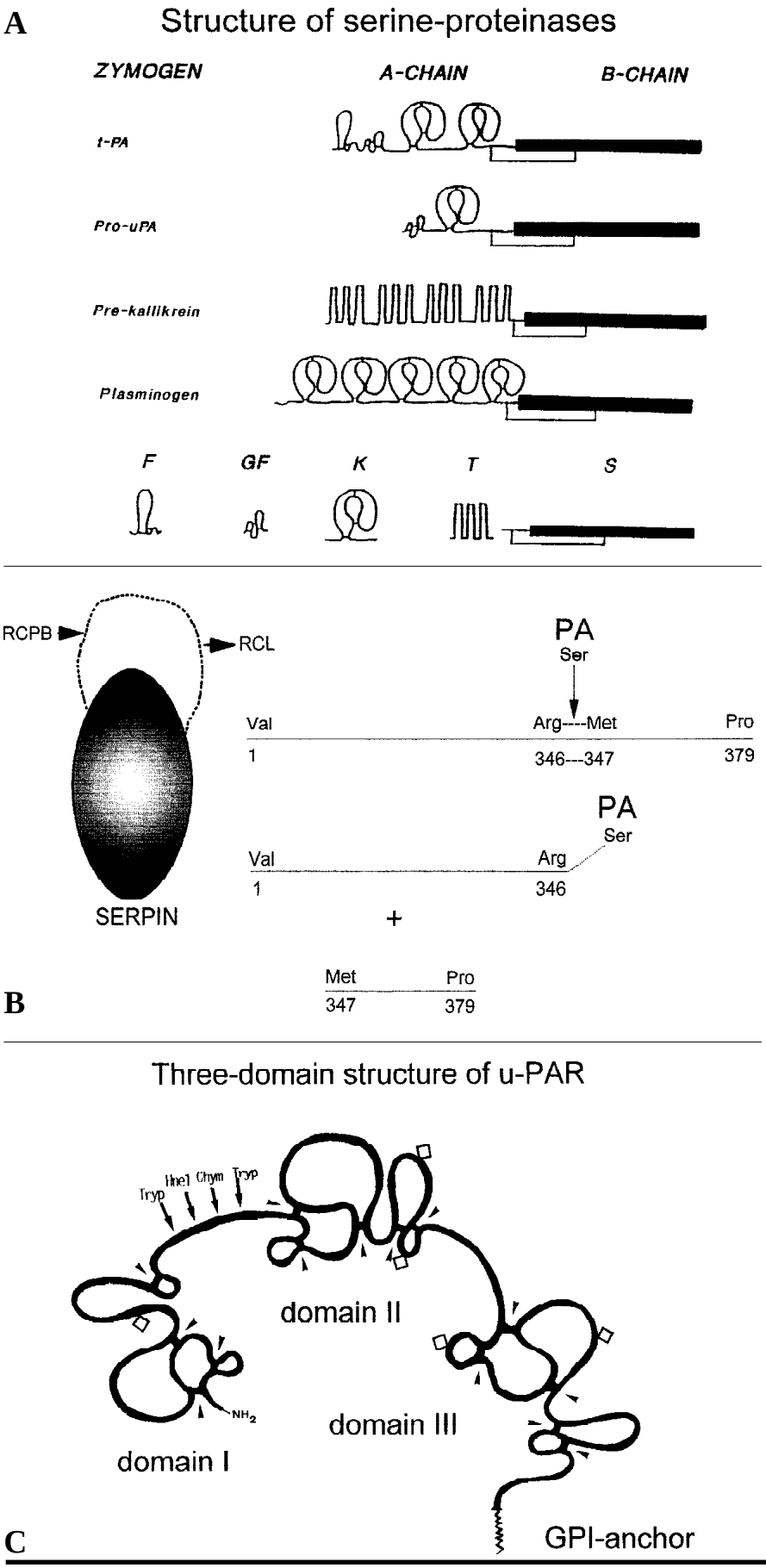

Fig. 1. The components of the fibrinolytic system: activators, inhibitors, and receptors.
A) Modular structure of some of the serine proteinases of fibrinolysis and coagulation. In the non-catalytic portion of the molecule, serine proteinases are arranged by the juxtapositioning of a limited number of modules: **F**, finger-type module; **GF**, growth factor module; **K**, kringle or ring-type module; **T**, tandem duplication module; **S**, the catalytic chain containing the serine proteinase site and the disulphide bridge, which connects the A and B chains after cleavage to the active form of the enzyme; **t-PA**, tissue-type plasminogen activator; and **pro-u-PA**, pro-urokinase-type plasminogen activator. The receptor-binding sequence of (pro-)u-PA is enframed within the GF domain (see text).
B) Basic structure of the inhibitors of the serpin family. **RCL**, reactive center loop; **RCPB**, reactive center peptide bond. On the right the mechanism of action of PAI-1 is shown. The serine-catalytic site of plasminogen activator (**PA**) cleaves the peptide bond between Arg346 and Met347. A new peptide bond is formed between the Ser of PA and Arg 346 (this means that the inhibitor is irreversibly used in the inhibition process). Finally, the Mr 4200 Met347-Pro379 peptide is released from the complex.
C) The structure of human u-PAR. The cysteine residues are joined by a black bar, indicated by the **arrowheads**. u-PAR has a three-domain structure, with inter-domain linker regions. The linker region between domains I and II shows the cleavage sites for some proteases: trypsin (**tryp**), human neutrophil elastase (**Hnel**), and chymotrypsin (**Chym**). The receptor may be differentially glycosylated and the diamonds in the figure indicate potential attachment sites for the N-linked carbohydrate side-chains (one site in domain I, two sites in domains II and III). Domain I is the u-PA-binding domain, while domain II interacts with vitronectin, β1 and β2-integrins and vitronectin (VN).

ess was already proposed at the beginning of this century (7). This idea has won increasing acceptance in recent decades and now there is no doubt that u-PA-mediated plasminogen activation is critical to the invasion process, as has been shown in many model systems both *in vitro* and *in vivo*.

We will review the data on the biochemistry, cell biology and molecular biology of various molecules of the fibrinolytic system useful to understand the implications of the u-PA/plasmin system in joint diseases.

Enzymes of the u-PA/plasmin system

Plasminogen and pro-u-PA are the zymogens of two important serine proteinases of the fibrinolytic system, plasmin (PL) and u-PA. The zymogens of the serine proteinases of both the coagulative and the fibrinolytic systems are characterized by the presence of long non-catalytic sequences (A chains) linked to a sequence which is homologous to trypsin (i.e., the portion endowed with the catalytic site, also called the B chain) (24). The serine proteinase A chain is composed of fixed stereomodules that may be subdivided into four fundamental types: module K or the "kringle"-type (a Scandinavian term referring to a ring-shaped cake); module F (finger-shaped); module GF, homologous to the epidermal growth factor; and module T, characterized by tandem repetitions (Fig. 1a). The u-PA A chain is characterized by a GF and a K module, and the plasminogen A chain by five K modules. These different modules correspond to autonomous "miniproteins" fused with the protease sequence of the B chain at different stages of their evolution. This modular organization provides the sequence- and stereospecificity needed for interaction with binding sites (8).

Plasminogen (90,000 Mr) is synthesized by the liver. The conversion of plasminogen to the two-chain PL occurs by cleavage of the peptide bond Arg560-Val561, which results in the formation of the non-catalytic A chain and the catalytic B chain, which are held together by a disulphide bridge (Fig. 1a). This conversion is catalyzed by u-PA, t-PA and some bacterial enzymes (for a review, see 9). PL has broad substrate specificity and degrades many ECM proteins, including fibronectin, fibrinogen, fibrin and vitronectin (VN) (for a review, see 3).

The single-chain pro-u-PA has an Mr of about 54,000, is released from many cells and is converted to the active form - the two-chain u-PA - by cleavage of the peptide bond Lys158-Ile159, which results in the formation of a disulphide-linked A and B chain serine proteinase (Fig. 1a). Such cleavage is performed mainly by PL, but other proteases have been reported to activate pro-u-PA, at least *in vitro* (for a review, see 8). Plasminogen is the main substrate of u-PA (3), but other substrates have been identified: hepatocyte growth factor/scatter factor (HGF/FS) and macrophage stimulating protein (MSP), which have considerable sequence similarity with plasminogen and acquire growth factor and motogenic activities after cleavage by u-PA (10). Most of this enzyme circulates as a complex with PAI-1 (11).

Inhibitors

Serine proteinase inhibitors (serpins) include different molecules which are specific for all the members of this enzyme family. The plasminogen activator/plasmin system is specifically inhibited by plasminogen activator inhibitor-1 (PAI-1), plasminogen activator inhibitor-2 (PAI-2) and α2-anti-plasmin (α2AP).

The interaction of α2AP (Mr ≈ 70,000, plasma concentration 1 μM) with PL is a two-step process (9). In the first step, the C-terminal region of the inhibitor interacts non-covalently with kringle 5 (K5) of PL, thereby adapting the inhibitor reactive site Arg 354-Met 355 of α2AP to the serine residue in the active site of PL. This reactive center peptide bond, which is a pseudo-substrate for the enzyme, is enframed within the so-called reactive center loop (RCL) (Fig. 1b). In the second step the serine residue cleaves this peptide bond, giving rise to a 11,000 Mr α2AP C-terminal fragment and to a 150,000 Mr covalent complex which is probably held together in a 1:1 stoichiometry by the newly formed Ser plg-Arg α2-antiplasmin bond (12, 13).

PAI-1 and PAI-2 equally inhibit u-PA and t-PA. Inhibition occurs in a single step which involves the serine active site of the enzyme and the reactive center peptide bond in the reactive center loop of both PAIs. The pseudo-substrate peptide bond is Arg 346-Met 347 for PAI-1 (Fig. 1b) and is Arg 358-Thr 359 for PAI-2. PAIs and PAs are also bound in a 1:1 stoichiometry involving a covalent bond between the Ser of PA and the Arg of the PAIs. PAI-1 is present *in vitro* in an inactive conformation which is reactivated following denaturation and refolding (12, 13). Active PAI-1 can interact with VN, which in turn stabilizes PAI-1 in the active conformation (14).

Other plasminogen activator inhibitors include protease-nexin and protein C inhibitor, whose pseudo-substrate bonds react with many serine proteinases (15, 16). In cultured cells, PAI-1 is uniformly distributed on the surface of culture dishes in association with VN (17).

Receptors

u-PAR was identified in the mid 1980s as a cell surface high affinity-binding site for the A chain of u-PA (18). The cDNAs encoding for human, murine, bovine and rat u-PAR have been cloned and sequenced. The carboxyterminal 31 residues contain the attachment site for a glycosylphosphatidylinositol (GPI) moiety which anchors u-PAR to the cell membrane (19). Due to its high cysteine content, the molecule is spatially organized in an internal triple repetition of cysteine spacing, resulting in the formation of three homologous, independently folded domains (domains 1, 2, 3 from the N-terminus) (20) (Fig. 1c). Five glycosylation sites are present in u-PAR, one of which is proximal to the amino-terminal binding domain; glycosylation can thus heavily affect ligand binding affinity (21).

u-PAR N-terminal domain 1 is the ligand binding site for u-PA (22), which in turn interacts with the receptor by a 7-aminoacid long residue of the growth factor domain (23). However, a strong contribution to ligand-receptor affinity is also provided by inter-domain interactions with domains 2 and 3 of u-PAR, whose function has not yet been clarified (24). The Kd of u-PA/u-PAR is in the range of 0.1 - 0.5 nM (8).

Other ligands interact with u-PAR: VN binds u-PAR with strong affinity (Kd, 0.2

to 2 nM), probably interacting with domains 2/3, while the u-PAR agonists (u-PA, pro-u-PA, u-PA A-chain, u-PA/PAI-1 complex) further promote binding (25, 26). Converging evidence points to the interaction of u-PAR domains 2/3 with the β2-and β1-integrins and such interactions have been shown to inhibit β1-integrin binding to fibronectin, thereby disrupting ECM integrity (27). In cultured cells u-PAR is selectively localized at cell focal contacts, where integrins are also accumulated (28, 29).

Both plasminogen and PL bind to cells with low affinity and high capacity by their lysine binding sites (LBS), indicating the presence of cell surface receptors (30). LBS are enframed within the kringle domains and interact with many proteins endowed with carboxy-terminal lysines, such as α-enolase (31) and annexin II (32), as well as glutaraldehyde 6-phosphate dehydrogenase (33). Amphoterin, a protein isolated from the brain, although exhibiting a high affinity for plasminogen/plasmin, represents a receptor without a carboxy-terminal lysine (34). Moreover, both gangliosides (35) and glycosaminoglycans (36) can bind plasminogen/plasmin, thereby contributing to the high capacity of cell surfaces for such ligands. Thus, the problem of identifying homogeneous properties for plasminogen/plasmin binding proteins on the cell surface remains unresolved at present, allowing such binding sites to be defined only operatively on the following bases: (i) their low affinity for the ligand (Kd, 100-2000 nM); (ii) their high density (from 10^6 to 10^7 PG molecules bound / cell); and (iii) their ubiquitous distribution (30).

Internalization of receptor-bound u-PA

Although u-PAR-bound u-PA can be internalized in a time-dependent manner at 37°C (37), upon interaction of PAI-1 with u-PAR-bound u-PA, the internalization of the u-PAR/u-PA/PAI-1 complex is greatly enhanced (37, 38). This process leads to the rapid lysosomal degradation of u-PA (37). PAI-2 and protease-nexin can substitute for PAI-1 (39-42).

The internalization of the u-PA/PAI-1 complexes requires previous binding to u-PAR. It is also known that additional receptors belonging to the low-density lipoprotein (LDL) receptor family are involved in the internalization process: the α2-macroglobulin receptor (α2MR), also called LDL receptor-related protein (LRP); gp330; and the VLDL receptor (VLDLR) (for a review, see 43). The combined roles of these receptors in mediating u-PA internalization is not yet well understood, but experimental evidence indicates that such receptors are all able to internalize u-PA/PAI-1 complexes, independently of previous binding to surface u-PAR. When the u-PA/PAI-1 complex is bound to u-PAR, u-PAR is internalized together with the u-PA/PAI-1 complexes, and is recycled to the cell membrane together with α2MR, LRP, gp330 and VLDLR (44). Internalization of the u-PA/protease-nexin and u-PA/protein C inhibitor complexes follows the same pathways (42, 45).

Functional interactions of the u-PA/plasmin system with vitronectin

As mentioned above, the Mr 78,000 glycoprotein VN interacts with the plasminogen activator/plasmin system. It is produced by the liver, its serum concentration is 4 μM and it is also present in the ECM. From the N-terminal end a somatomedin B domain, an integrin-binding sequence (RGD), a collagen-binding domain and 2 hemopexin-like regions (Fig. 2) have been identified (14, 17). The three-dimensional structure of VN may be either closed or extended: the closed conformation is typical of circulating VN, while the extended conformation is found in ECM-associated VN. PAI-1 and u-PAR may compete for binding to the somatomedin B domain in the extended conformation, while only PAI-1 binds to the closed conformation (26, 46). Many integrins bind to the RGD sequence of VN in both the closed and extended conformations and their binding is competed for by PAI-1. A low-affinity interaction has also been described between the hemopexin domains of VN, u-PA and plasminogen.

u-PAR-dependent cell invasion and collagenases

u-PAR binds either u-PA or its zymogen pro-u-PA. Both forms can interact with the receptor after their production either by u-PAR-expressing cells (autocriny) or by other cells (47). Morphological and biochemical evidence indicates that (pro)-u-PA-saturated u-PAR is concentrated at the focal adhesion sites of cul-

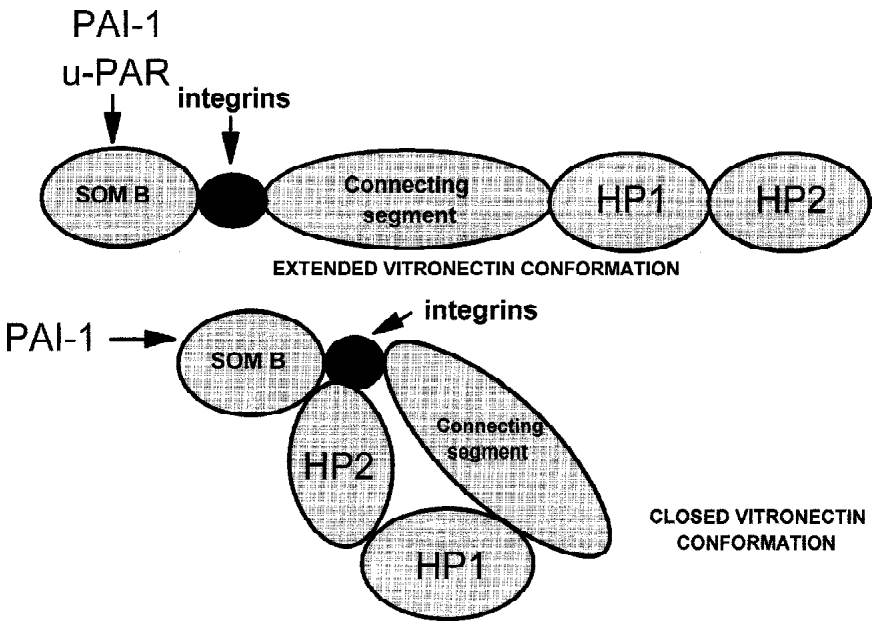

Fig. 2. The structure of vitronectin. Vitronectin consists of five different domains and may be present either in an extended or a closed conformation. The NH_2-terminal somatomedin B domain (SOM B) can bind PAI-1 and u-PAR when it is in the extended conformation, while it binds only PAI-1 when the closed conformation prevails. The binding site for integrins contains typical RGD sequences. The connecting segment shows affinity sequences for collagen. HP1 and HP2 represent the carboxy-terminal hemopexin-like domains.

tured cells, where integrins are also accumulated and where integrin cytoplasmic domains interact with actin filaments (28, 29). It is possible that the primary force driving u-PAR at the focal contacts is the presence of VN on the adhesion substrate, whose somatomedin B domain shows u-PAR affinity. Moreover, there is considerable evidence of u-PAR interaction with the β1- and β2-integrins, which also are abundant at focal adhesion sites (for a review, see 46).

The *in vitro* conversion of pro-u-PA to u-PA is performed by many enzymes in addition to PL: plasma kallikrein and blood coagulation factor XIIa (48), two trypsin-like ovarian tumors proteinases (49), cathepsin B (50), cathepsin L (51), nerve growth factor-γ (52), and prostate-specific antigen (53). Little is known about pro-u-PA activation *in vivo*, however, where PL seems to play a crucial role. Activation occurs at focal contacts (54) and u-PAR-bound pro-u-PA is activated more rapidly than fluid-phase pro-u-PA (55).

Both pro-u-PA and plasminogen accumulate on the cell surface upon interaction with the receptors and binding sites described above. The amplification of the multi-enzyme cascade starts with the activation to PL of receptor-bound plasminogen by u-PAR-bound u-PA. Once formed, PL can degrade either directly or indirectly, through the activation of secreted pro-matrix metalloproteinases (pro-MMPs), the greater part of the ECM molecules, thereby favouring the movement of cells across anatomical barriers (56).

Many findings indicate that cell-surface-associated PL, generated directly on plasminogen binding sites by u-PAR-bound u-PA, is an active enzyme that catalytically promotes the destruction of a broad range of ECM proteins. In this respect, the functional importance of cross-talk between the plasminogen activator/plasmin system and the MMPs system is becoming increasingly clear. At least twelve different MMPs have been described; among these gelatinase A (MMP-2), which degrades gelatin, elastin and collagen type IV, is produced by stromal cells, including the fibroblasts surrounding malignant tumors (57). Uncharacterized high-affinity binding sites for MMP-2 have been described on the surface of malignant cells (58). Immunolocalization at the plasma membrane of interstitial collagenases has been reported, which suggests the existence of specific receptors for other members of the MMPs family both on neoplastic and non-neoplastic cells (59).

Gelatinase A is secreted as a pro-enzyme (pro-Gel-A) and, once associated to the plasma membrane (58, 60), its activation is triggered by membrane-type MMPs (MT-MMPs), i.e. recently identified cell membrane integral enzymes (61) endowed with an intracellular and a transmembrane domain and an extracellular domain containing the catalytic site. As shown in Figure 3, the activation of pro-

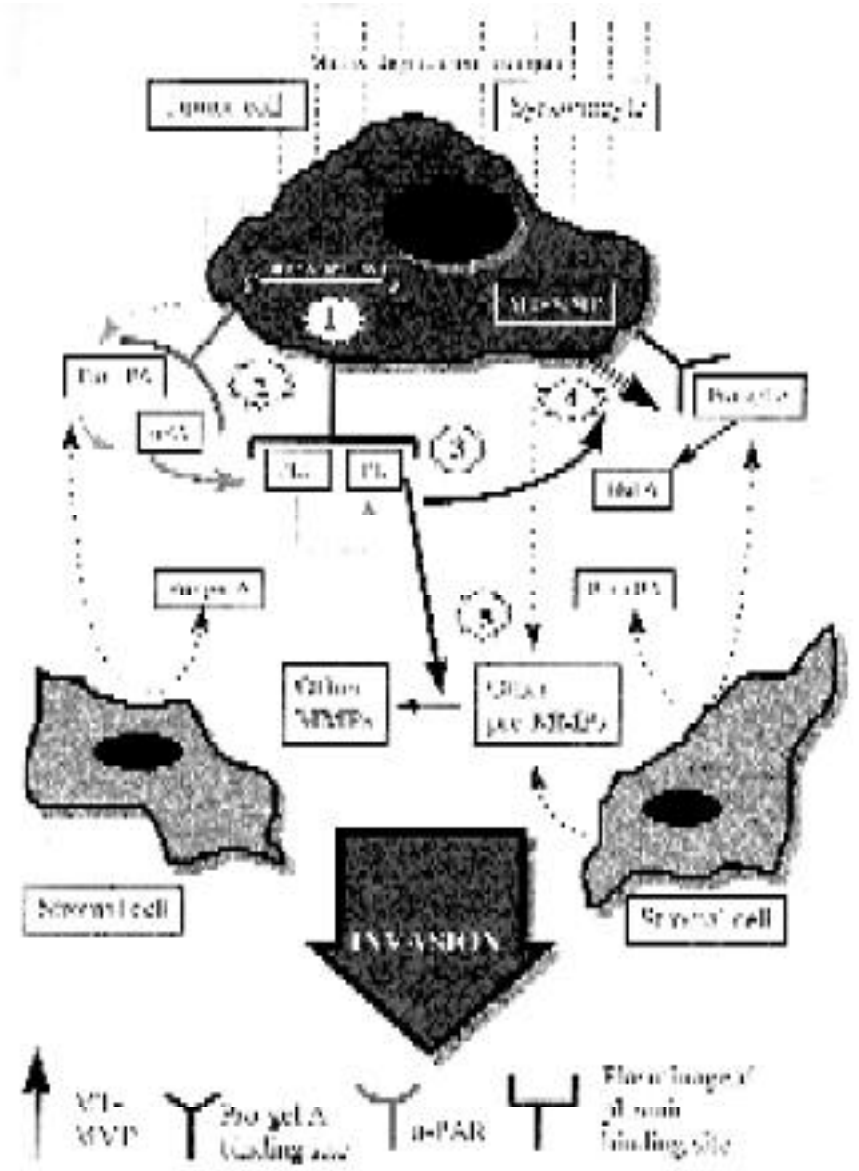

Fig. 3. Surface-driven proteolysis of invasive cells (such as cancer cells, or activated synoviocyte in rheumatic pathologies). Two classes of membrane-associated proteases cooperate in the degradation of ECM molecules. The plasminogen activator/plasmin system includes the (pro-)urokinase-type plasminogen activator (**pro-u-PA** and **u-PA**) and its receptor, plasminogen (**PLG**) and plasmin (**PL**), as well as their binding sites on the cell membrane. The matrix metallo-proteinase (**MMPs**) system includes pro-gelatinase A (**Pro-gel-A**) and its as yet unidentified binding site, **Gel A**, the membrane-type matrix metallo-proteinase (**MT-MMP**), and a large series of pro-MMPs released into the ECM. PL, Gel A and other MMPs appear to be active in ECM degradation. The protease cascade and the steps that control activation are: (**1**) the synthesis of u-PAR; (**2**) the interaction of (pro-)u-PA with u-PAR; (**3**) the activation of PLG to PL; (**4**) the PL-dependent activation of MT-MMP, which in turn activates pro-Gel A to Gel A; and (**5**) the PL-dependent activation of pro-MMPs to MMPs.

Gel-A occurs following the interaction of the pro-enzyme with a cell surface activator (MT-MMP) in a complex PL-dependent fashion (62). TIMP-2 (tissue inhibitor of metalloproteinase-type 2) interacts with MT-MMP and receptor-bound pro-Gel-A interacts with TIMP-2/MT-MMP complex at its carboxyl-end, undergoing activation to Gel-A.

But how is the activator (MT-MMP) activated ? A possible answer lies in the aminoacid sequence lying just upstream of the amino-terminus of MT-MMP (Arg-Arg-Lys-Arg), which makes this peptide such an excellent substrate for serine proteinases such as PL or u-PA. This sequence is also present in other members of the MMPs family that can be activated by PL and is absent in pro-Gel-A and its analogs, which are resistent to direct PL activation. It is therefore likely that pro-Gel-A activation may be the result of a cell surface proteolytic cascade involving the activation of MT-MMP by membrane-associated PL and/or u-PA. The activated MT-MMP can thus interact with TIMP-2, forming a complex that binds and activates pro-Gel-A. Therefore, the activation of both membrane-associated and fluid phase MMPs are also dependent on the membrane plasminogen activator/PL system (Fig. 3).

Although many studies have underlined the importance of u-PAR-bound u-PA for plasminogen activation, leading to localized ECM destruction at the immediate cell periphery, u-PAR-independent pathways of plasminogen activation may also exist. In fact, experiments with animals which have been knocked out for the u-PAR gene (u-PAR$^{-/-}$) suggest that u-PA can act independently of u-PAR, at least in functions where the lysis of fibrin clots are concerned (63). On the other hand, the physical association between u-PAR and the β1 integrins appears to have functional consequences in terms of u-PA-independent invasion. In fact, the direct association of u-PAR domains 2/3 with β1 integrins inhibits the interaction between integrins and their ECM ligands (64). Therefore, this remains an alternative mechanism to modulate cell attachment to ECM.

Plasmin generation-independent functions of the u-PA/u-PAR system and signal transduction

In addition to its function in the fibrinolytic system, several lines of evidence indicate that the interaction of u-PA with u-PAR elicits a complex series of events ranging from chemotaxis and chemokinesis (65-70) to cell multiplication (70-75), differentiation (76, 77) and the autocrine secretion of u-PA (78) (Fig. 4). Such effects have been observed in all cells exhibiting u-PAR on their membrane, including endothelial cells, monocytes, epidermal cells, fibroblasts, osteoblasts, synoviocytes, and chondrocytes. In most cases, the effects following u-PA/u-PAR interaction do not require the plasminogen activation activity of the enzyme and occur with both native u-PA and the u-PA A-chain. The only exception is u-PA-dependent cell proliferation, which requires an intact catalytic site (though there is also evidence to the contrary, at least in one cell line) (74). Whatever the case, in u-PA-dependent cell proliferation the substrate of u-PA catalytic activity is not plasminogen, since it occurs even in the presence of specific inhibitors of plasminogen activation. Thereby, the interaction could be considered as similar to that occurring betweenpolypeptide chemotactic/growth factors and their membrane receptors. Such interaction requires transduction at the level of the cell membrane. With u-PAR the problem is complicated by the GPI-anchor, which is the only structure linking the receptor to the plasma membrane: such anchorage cannot transduce by itself nor interact with other transducing molecules, such as the protein tyrosine kinases or G-proteins, which are confined to the inner leaflet of the plasma membrane. Therefore, the existence of an adaptor molecule has been suggested, able to couple extra-cellular contact with intra-cellular transducing mechanisms. Possible candidates are caveolin and the β2 integrins LFA-1 and CR3; however, only indirect evidence of interaction has been provided, such as co-localization by confocal microscopy, antibody-induced co-capping and immune precipitation.

Some papers have recently addressed the topic of u-PA/u-PAR signal transduction events. These include: the serine phosphorylation of cytokeratins (69); the tyrosine phosphorylation of a 38 kDa protein (79); the association of u-PAR with a long series of tyrosine-kinases of the src family (p60^{fyn}, p53/p56^{lyn}, p56/p59^{hck}, p59^{fgr}) (80, 81); the β1, β2 and β3 integrins (27, 80, 82); the *de novo* synthesis of diacylglycerol by the tyrosine-phosphorylation-dependent translocation/activation of glucose transporters (83, 84); mechanical coupling to the cytoskeleton (85); cAMP formation (86); the activation of inositol phosphate turnover, induction of Ca influx and release of Ca from intra-cellular stores (87); and c-fos gene expression (88). On the basis

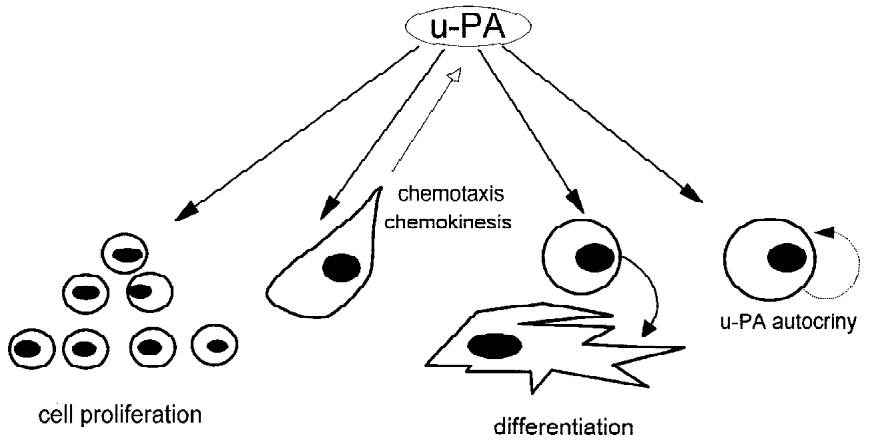

Fig. 4. Plasminogen-independent activation activities of u-PA. For details, see text. With the exception of u-PA-dependent cell proliferation, all of the activity depicted in the figure is elicited by either u-PA or u-PA-ATF, indicating that the presence of catalytic activity is not required. Cell proliferation is plasminogen-independent, since it requires u-PA catalytic activity but can occur even in the presence of plasmin inhibitors. The substrate involved is still unknown.

of a preferential localization of GPI-anchored molecules (including u-PAR) in the plasmalemmal vesicles referred to as caveolae, which much evidence suggest to be signalling organelles, recent data has been obtained indicating that u-PA/u-PAR also utilizes the caveolae-associated JAK/STAT1 pathway for signaling (89, 90).

On the basis of the available data it is impossible at present to depict a likely sequence for the multiple signal transduction pathways utilized by the u-PA/u-PAR system. Both u-PA and plasmin are involved in the activation of latent growth factors. By means of a single proteolytic cleavage, the PA/PL system activates HGF/SF, MSP, TGF-β and b-FGF (see above and ref. 46). Therefore, every attempt to study the signal transduction mechanism triggered by u-PA/u-PAR interactions following exogenous addition of the ligand should take into consideration the presence of even trace amounts of such powerful growth factors in the cell model system under study, whose activation could account for the observed transductions.

The fibrinolytic system in inflammatory joint diseases

Fibrin and fibrinolysis

Plasminogen activators. Fibrin and the fibrinolytic system have been implicated in rheumatic pathologies since the 1960s. Fibrinogen is not detectable in the synovial fluid of healthy human joints. In 1961 Harrold (91) suggested that fibrinogen entering the inflamed joint becomes the substrate for two competing reactions: fibrin formation by thrombin or fibrinolysis by plasmin. It was observed that the amount of intra-articular fibrin deposition reflects different grades of joint inflammation and that continual and unresolved fibrin deposition may provoke permanent, chronic joint damage (92). Indeed, the finding that plasminogen and plasminogen activator were produced by the synovial membrane and capsule of normal and abnormal human joints, and possibly also by leukocytes of the inflammatory exudate, indicated that secondary fibrinolysis also occurs in the inflamed joint (93). Thus, fibrin formation and fibrinolysis represent the opposing aspects of a two-faced coin in the pathogenesis and maintainance of joint lesions.

The secretion of plasminogen activator and collagenases by rheumatoid and non-rheumatoid synovial cells in culture was subsequently shown (94, 95). After demonstration of the role of plasminogen activators in the endogenous activation of latent collagenases (95), intra-articular plasminogen activation has been envisaged as a possible trigger of cartilage destruction, allowing invasion of the synovial pannus in RA. In this setting, after its conversion from plasminogen, plasmin could activate the latent collagenase bound to collagen fibrils, and the combined effects of collagenase and plasmin on extracellular connective tissue could facilitate the migration and growth of capillaries into the tissues, thereby extending the area of connective tissue destruction or remodelling (see Fig. 3).

Other studies then demonstrated that the supernatant of cultured mononuclear cells, which are widely represented in the inflammatory exudate of arthritic joints, stimulate the release of plasminogen activator and latent collagenases from synovial cell monolayer cultures (96, 97). Thus, the similarity between rheumatoid synovial cells and cancer cells in terms of the release of enzymes able to degrade extracellular matrix led to the identification of common invasive properties between the rheumatoid pannus and malignant cells (98, 99).

Since these early observations many authors have reported an elevation of plasminogen activators in RA joints compared with both normal and OA joints, and have also reported that in most cases the urokinase-type plasminogen activator (u-PA) was the PA involved (100-112). In osteoarthritis synovial inflammation is a common phenomenon. Histologic grading has clearly established that OA-associated synovitis can range from low-grade to moderate to severe inflammatory reactions.

Synovial inflammation in OA is probably a secondary phenomenon linked to various factors. Cartilage breakdown products (113,114), including apatite and calcium pyrophosphate dihydrate crystals (115-117), may be released from damaged cartilage and eventually be phagocytosed by synovial cells and macrophages, thereby initiating an inflammatory reaction. Inflamed synovium in turn may contribute to cartilage destruction (118).

Martell-Pelletier *et al.* (119) have shown that synovial cell-derived plasminogen activators account for the serine protease activity detected in human OA synovium. Such activity, related to the presence of u-PA, is more than five-fold higher in OA than in normal synovium (120). The production of u-PA by inflamed synovium may be controlled by nonsteroidal antiinflammatory drugs (101, 121, 122). In a study by Kikuchi *et al*. (123) u-PA and MMPs levels in the articular cartilage of OA and RA patients were compared the levels in subjects with no history of joint diseases. It was found that in OA and RA patients both the serine proteinase and MMPs systems are expressed at high rates, indicating that in joint pathologies characterized by cartilage remodelling and/or invasion there is an amplification of the main protease systems involved in ECM degradation.

Plasminogen activators/inhibitors. Lowered fibrinolytic activity at the sites of chronic inflammatory infiltrates and of fibrin deposits in the joint were first observed by van de Putte *et al.* (124). These were linked to the presence of inhibitors of fibrinolysis which could be responsible for the prolonged persistence of such deposits in the rheumatoid joint.

Following this report, contradictory data appeared in the literature. Mochan *et al.* (100) failed to find evidence of a modification in plasminogen activator inhibitors in the synovial fluid of patients with RA. It was then demonstrated that phorbol myristate acetate added to human synovial fibroblast cultures caused a dose-dependent increase in the production of PAI-1, coupled with a decrease in plasminogen activator production (125).

Intra-articular levels of u-PA/PAI complexes were shown to increase in RA with respect to OA and seronegative spondyloarthropathy (SAA), and to correlate with clinical parameters (104). u-PA, PAI and u-PA/PAI complexes in the synovial fluid of arthritic joints were all shown to be higher than plasma levels in the same patients or in heathy donors

(105, 106). Saxne *et al*. (107) reported an increase of leukocyte-derived PAI-2 and of PAI-1 in RA synovial fluid compared to OA synovial fluid. A direct correlation of PAI-2 with granulocyte infiltrate, as well as an elevation of PAI-1 and α2-macroglobulin, were reported by Blaser *et al*. (108) and Belcher *et al*. (109) in inflamed joints. Synovial tissue extracts from RA patients have shown higher levels of PAI-1 and PAI-2 than synovial tissues from OA donors (110). Finally, cultured synovial cells show a higher production of PAI-1 with respect to OA and normal synoviocytes (126).

Receptors for u-PA. All resident articular cells have been shown to expose bona fide receptors for u-PA. The first observation of the presence of u-PAR in human synovial cells was obtained in our laboratory by the transmission electron microscopy of a gold:u-PA complex and by the radioligand binding of ^{125}I-u-PA (127). Scatchard analysis of the binding data indicated the presence of 95 to 230 x 10^3 u-PAR/cell and a Kd of 1.8 x 10^{-9} M. The up-regulation of u-PAR in RA synoviocytes compared to u-PAR in OA and normal donors was then reported by Ronday *et al.* (110) and Szekanecz *et al.* (128), in addition to the degradation of non-mineralized and mineralized bone matrix by synovial u-PAR-bound u-PA (111). Busso *et al.* (112) have shown the up-regulation of u-PAR and PAI-1 in RA synovial tissue. While we could confirm the increased production of PAI-1 by RA synovial cells, we were unable to demonstrate any differences in u-PAR between RA, OA and normal synoviocytes (125). We then showed that the u-PA/u-PAR interaction stimulates the chemotaxis, chemoinvasion and cell proliferation of human synovial cells from normal donors (71).

Chondrocytes also express u-PAR on their membranes (127), the amount being highly variable in both normal and OA chondrocytes. However, cells from normal donors expose fewer receptors than cells from OA patients (4.93 - 67.3 x 10^3 receptors/cell and Kd 0.69 - 6.47 x 10^{-9} M in normal chondrocytes; 77.9 - 488.7 x 10^3 receptors/cell and Kd 1.55 - 71.9 x 10^{-9} M in the chondrocytes of OA patients). In addition, chondrocytes undergo u-PAR-dependent chemotaxis, chemoinvasion and proliferation (71).

The receptor for u-PA was first demonstrated on a myelomonocytic leukemic cell line (U-937 cells) (18). Normal peripheral blood and intra-articular monocytes were also shown to express u-PAR, which accounts for the increased proteolytic activity of synovial monocytes from patients with RA with respect to OA patients and healthy volunteers (129). Monocyte/macrophage-associated u-PAR and the resulting cell membrane-associated fibrinolytic activity can be modulated by nonsteroidal antiinflammatory drugs (130).

Neoangiogenesis sustains both tumor enlargement and synovial pannus proliferation and requires endothelial cell migration and proliferation. These events are linked to the modification of both the adhesive properties of endothelial cells with extracellular matrix components and cell-cell interactions. A major role in this context is played by the plasminogen activator system and experimental evidence has shown that the u-PA/u-PAR interaction elicits endothelial cell chemotaxis and chemokinesis *in vitro* (65), as well as angiogenesis *in vivo* (131, 132).

Sources and regulation of PA and PAI inhibitors in the synovial fluid

Non-inflamed synovium is generally considered to consist mainly of macrophage-like (type A) and fibroblast-like (type B) cells. Therefore, it is likely that the sources of plasminogen activators and PAI in the OA and RA synovia are the resident articular cells (monocyte/macrophages, synovial fibroblasts, chondrocytes) and inflammation-associated cells (monocytes and polymorphonuclear cells, PMN).

PA/PAI production by the monocyte. Several growth factors which control hemopoiesis have been identified in the synovial fluid of RA: granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage-CSF (M-CSF, or CSF-1), granulocyte-CSF (G-CSF), and interleukin 3 (IL-3, also known as multi-CSF), which cause the proliferation and differentiation of many pluripotent progenitor cells (133-135). Such growth factors stimulate u-PA activity and the m-RNA level of human monocytes *in vitro* (136) (Fig. 5). Under appropriate stimuli, such as by LPS, monocytes can themselves produce GM-CSF and G-CSF, ini-

Monokine- and CSF-mediated cross-talk among macrophages, chondrocytes and synoviocytes for the control of u-PA production

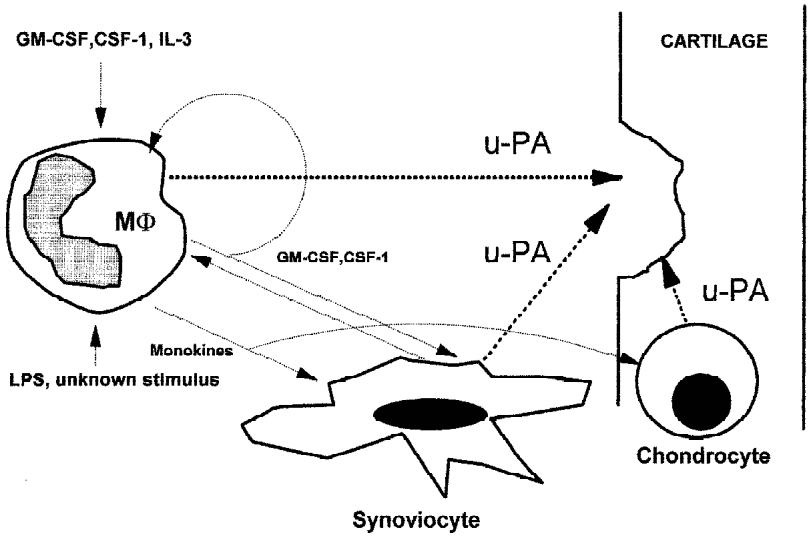

Fig. 5. The cytokine-mediated cross-talk among macrophages, chondrocytes and synoviocytes. The concerted action of a long series of cytokines leads to the hypersecretion of u-PA by macrophages (**MΦ**), synovial cells and chondrocytes. The final lesion is probably mediated by u-PAR-bound u-PA. The presence of high intra-articular levels of u-PA also takes part in macrophage recruitment within the inflammed joint, cartilage remodelling-related chondrocyte movements, and synovial pannus proliferation and invasion into underlying cartilage. As described in the text, synovial pannus angiogenesis is also largely dependent on the u-PA/plasmin system.

tiating an autocrine loop which leads to the enhanced production of u-PA (137). The same triggers also induce monocytes to secrete interleukin 1 (IL-1) and tumor necrosis factor α (TNFα), which in turn induce the production of u-PA, GM-CSF and G-CSF from synoviocytes (138) and chondrocytes (137). Thus, all resident articular cells (monocytes, synoviocytes and chondrocytes) are able to produce at the same time haemopoietic growth factors and plasminogen activators, in a sort of amplification cascade which could result in increased joint u-PA activity in arthritis (Fig. 5).

There are no reports of PAI-1 production by monocytes, but they can express PAI-2 after stimulation with LPS (139). Macrophages can also be induced to express a procoagulant activity which promotes fibrin deposition (140, 141), thereby enhancing both fibrin deposition and removal. In addition, PMN contain u-PA in their intracellular granules (142) and PAI-2 in another compartment (143); thus, stimuli that induce PMN degranulation will contribute to enhance u-PA levels to the sites of inflammation.

PA/PAI production by synoviocytes. Synovial fibroblast-like cells represent yet another potential source of u-PA production in arthritic joints. IL-1, which is mainly produced by monocytes, is able to induce u-PA activity in human joints, a property that it shares with retinoic acid (144-146). Since the monokine-dependent induction of u-PA expression in synovial cells can be inhibited by nonsteroidal antiinflammatory drugs, prostaglandins have been implicated in the coupling of IL-1 stimulation with u-PA production (147, 148). Thus, in the inflamed joint the IL-1-dependent increase of u-PA is both direct via the stimulation of synovial fibroblast-like cells and indirect via the action of synoviocyte-derived GM-CSF on monocyte u-PA production (Fig. 5).

Oncostatin M, another growth regulator which is produced by activated T-lymphocytes and monocytes, also stimulates u-PA activity in human synovial fibroblasts (149). RA synovium (compared with the synovium of patients with OA or joint trauma) has an increased number of macrophages and an increased expression/content of fibrinogen, tissue factor, coagulation factor XIII, u-PA and α2-plasmin inhibitor (150) These observations suggest that in the inflamed synovium ongoing extra-vascular tissue fibrin formation and dissolution are taking place which correlate with the degree of inflammation and the macrophage content. Although PAI-1 is the main inhibitor, PAI-2 is also produced by human synoviocytes (137).

PA/PAI production by the chondrocytes. Even human chondrocytes can produce and release PA into joints. McGuire-Goldring *et al*. (151) have shown that purified preparations of IL-1 derived from human blood monocytes stimulate the production of prostaglandin E and plasminogen activator by human articular chondrocytes. IL-1, TNF-α, and all-trans retinoic acid can all stimulate the u-PA secretion by chondrocytes (152), although there is also experimental evidence indicating that u-PA production by chondrocytes may be independent of IL-1 (153). Levels of PA and plasmin were found to be significantly elevated in the OA knee cartilage of dogs subjected to sectioning of the anterior cruciate ligament of their right knees (154). It is interesting that chondrocytes isolated from the growth zone of rat chondrocostal cartilage produce matrix vesicles and plasma membranes containing plasminogen activators and MMPs that are differentially regulated by vitamin D metabolites (155). The addition of proteolytically generated fibronectin fragments to cultured human cartilage induces the release of elevated levels of u-PA, a powerful activator of pro-stromelysin-1 which in turn causes extensive cartilage destruction (156). Moreover, chondrocytes produce high amounts of PAI-1 (137) that decrease upon stimulation with IL-1 (153).

Vitronectin is present in synovia

Vitronectin, the adhesive blood protein which modulates the fibrinolytic system by its capacity to interact with PAI-1, plasminogen activators, u-PAR and plasminogen (46), has been described in the synovial tissue. In normal synovial tissue, both endothelial cells and synovial cells have been reported to express VN, which is located between the intimal, subintimal and vascular compartments of the synovial membrane (157). Rheumatoid arthritic synovia additionally express VN in infiltrating inflammatory cells, and RA synoviocytes produce VN *in vitro*, which inhibits plasmin generation (158). Furthermore, synoviocytes of OA and RA joints exhibit the αvβ3 VN receptor on their membrane, which suggests a functional interaction between these receptors and the adhesion molecule (159, 160). Surprisingly, in Rinaldi' study the proinflammatory cytokines TNF-α and IL-1β increased the expression of VN and its receptor on the synovial cells from OA patients, while the same molecules were down-regulated in synoviocytes from RA patients (159).

A new fibrinolytic scenario in the pathogenesis of joint inflammation

The most characteristic feature of RA is persistent inflammatory synovitis, which represents the pivotal event leading to cartilage destruction, bone erosion and subsequent joint deformities. Recent discoveries in the area of the regulation and activity of cell surface-driven fibrinolytic mechanisms allow us to hypothesise the following chain of events hinging upon the specific receptor for u-PA.

Within inflammed joints many growth factors and cytokines, as well as stimuli of unknown origin, share as their target monocytes, synovial cells and chondrocytes, thus creating a cytokine network which amplifies cell activation by paracrine and autocrine mechanisms (Fig. 5). These amplification loops lead to the hypersecretion of u-PA by resident and inflammation-associated cells, which could then cause joint lesions via the following mechanisms.

1. u-PA could interact with u-PAR on the surface of monocytes, synovial cells and chondrocytes, thereby initiating a cascade of plasminogen activation-dependent events. Cross-talk between the surface-activated plasmin and both receptor-bound and fluid phase pro-collagenases leading to collagenase activation in the immediate cell periphery could then cause the cartilage destruction and bone erosions associated with joint inflammation.

2. The u-PA/u-PAR interaction also trig-

gers plasminogen activation-independent events which we believe to be critical in the natural history of joint lesions. Chemotactic/chemokinetic effects exerted by u-PA on u-PAR-bearing cells could stimulate mononuclear phagocyte infiltration within the inflamed joint, in concert with other powerful chemotactic agents, at the same time providing the cell with an active proteolytic mechanism able to destroy the extra-cellular matrix. This same activity could stimulate synovial cell movement and infiltration on the articular surfaces, as well as chondrocyte motility related to tissue remodelling coupled with cartilage destruction.

At the same time, u-PA-dependent proliferative activity could represent an important cofactor in sustaining the growth of the synovial pannus and cartilage remodelling. In this context, the pro-angiogenic activity of the u-PA/u-PAR interaction could sustain the capillary growth which parallels synovial pannus proliferation (84). The u-PA system also affects cell adhesion through the direct binding of u-PAR to vitronectin in an RGD-independent fashion.

From this standpoint, particular attention should be concentrated on recent studies of integrin expression in synovial cells and their regulation by inflammatory cytokines. Indeed, it has been shown that the proinflammatory cytokines, especially IL-1β, increase the expression of α1-integrin in synovial fibroblasts and in macrophages cultured *in vitro* (161). This observation is very important if one considers the fact that VN interacts with cell-surface integrins. In turn u-PAR binds VN, thus providing a means for cell adhesion to take place, where matrix degradation mediated by u-PAR-bound u-PA will also occur.

In other words, the synoviocyte could exploit cytokine-induced integrin expression for subsequent cycles of cell attachment and detachment mediated by different domains of u-PAR: domain 1 is involved in u-PA-dependent ECM degradation, while domains 2 and 3 regulate adhesive interactions with integrin-bound VN, which provides a sort of pathway for cell invasion within inflamed tissues. Finally, the reported elevation of PAI-1 in inflamed joints could also be fitted into the present scenario: the PAIs can be regarded as extra-cellular, matrix-stabilizing compounds via their ability to block extra-cellular matrix proteolysis. This is an important requisite to provide cells with a substrate to favour cell movement. Therefore cell migration may be envisaged as a kind of “stop and go” signal provided by the alternating prevalence of matrix degradation and stabilization (162).

The present hypothesis suggests that the high fibrin content found in chronic synovitis, sometimes organized as “rice bodies” in the synovial fluid, is the final result of such alternating cycles, while fibrinolysis prevails at the beginning of the pathogenetic process.

The typical lesions of RA begin at the synovial membrane level. In the early phase, the pathogenetic process is ruled by exudative inflammation, with the presence of fibrinogen, fibrin and neutrophils within the inflamed joint. In this phase both inflammation-associated cells and synoviocytes, under the stimulus of cytokines and other pro-inflammatory factors, produce u-PA which interacts by autocriny and paracriny with u-PAR-bearing cells, as well as with plasminogen, in order to: (a) contribute to the recruitment of inflammatory cells in the joint, and (b) degrade fibrin clots.

Therefore, the fibrinolytic system serves two distinct functions in the early phase of RA: (1) to recruit defence cells within the joint and provide u-PAR-bearing cells with a proteolytic apparatus enabling them to invade the joint structures; and (2) to destroy a potentially dangerous fibrin-generated provisional matrix which could be exploited in a repair process leading to intra-articular fibrosis. In this scenario, the PA activity measured in RA synovia could be related to a “secondary fibrinolysis” process and thus acquire a positive prognostic significance.

In the late phase the pathogenetic process is dominated by the formation of granulation tissue, coupled with the proliferation of fibroblasts, vessels and synovium, the latter of which appears thickened and villous. In this phase there is little or no fibrin to be digested and the intra-articular u-PA, mainly provided by synovial cells and macrophages, serves plasminogen-dependent and independent roles, stimulating the proliferation of synovium and its invasion into the underlying cartilage. In this phase the intra-articular PA activity acquires a negative prognostic meaning.

Conclusions and prospects

Fibrinolysis is not only an event linked to the clearance of fibrin in the circulation. It is now clear that the fibrinolytic process plays a fundamental role in tissue invasion in different diseases and in angiogenesis.

In this review we have provided evidence that u-PAR constitutes one of the key players in the pathogenetic chain of inflammation-dependent damage in rheumatic joints. We have presented a molecular basis for earlier hypotheses, looking at the synoviocyte as a kind of "transformed cell" exhibiting proliferative and infiltrative activities which closely resemble the main properties of cancer cells. Since the behaviour of the synovial cell in joint inflammation is affected by factors other than genetic ones, we believe that synoviocytes, as well as chondrocytes, must be regarded as “innocent by-standers” which acquire invasive and proliferative properties under the influence of the inflammatory cytokine network originating in the inflamed joint. All of these observations lead us to consider the cell-associated fibrinolytic system as a final pathway that regulates the invasive, destructive and proliferative activities of synovial cells and chondrocytes both by itself and by the activation of pro-MMPs.

Therefore, the development of pharmacological tools able to control the plasminogen activation system in the late phase of articular inflammation may offer clinicians the possibility in the near future to profoundly modify the development of bone lesions and eventual joint ankylosis.

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