MULTIFUNCTIONAL ROLES OF PLASMIN IN INFLAMMATION

Studies of animal models on rheumatoid arthritis, multiple sclerosis, wound healing and infection

by

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PAPERS I-V
ABBREVIATIONS

\( \alpha_2 \)-AP \( \alpha_2 \)-antiplasmin
AIA antigen-induced arthritis
BBB blood-brain barrier
CAIA collagen type II antibody-induced arthritis
CD cluster of differentiation
CFA complete Freund’s adjuvant
CIA collagen type II-induced arthritis
CII collagen type II
CNS central nervous system
CSF cerebrospinal fluid
EAE experimental autoimmune encephalomyelitis
ECM extracellular matrix
EGF epidermal growth factor
FGF fibroblast growth factor
GM-CSF granulocyte/macrophage colony-stimulating factor
IFN interferon
Ig immunoglobulin
IgG immunoglobulin G
IL interleukin
kDa kilodalton
LIA local trauma-induced arthritis
MBP myelin basic protein
mBSA methylated bovine serum albumin
MHC II major histocompatibility complex class II
MMP matrix metalloproteinase
MOG myelin oligodendrocyte glycoprotein
MS multiple sclerosis
OM otitis media
PA plasminogen activator
PAI-1 PA inhibitor type-1
PAI-2 PA inhibitor type-2
PDGF platelet-derived growth factor
PF pars flaccida
PN-1 protease nexin 1
PT pars tensa
RA rheumatoid arthritis
serpin serine protease inhibitor
TGF transforming growth factor
TM tympanic membrane
TNF tumour necrosis factor
tPA tissue-type PA
uPA urokinase-type PA
uPAR uPA receptor
ABSTRACT

MULTIFUNCTIONAL ROLES OF PLASMIN IN INFLAMMATION

Studies of animal models on rheumatoid arthritis, multiple sclerosis, wound healing and infection

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Plasmin has been suggested to be involved in degradation of extracellular matrix (ECM) and tissue remodeling during a number of physiological and pathological processes. The aims of this thesis were to study the functional roles of plasmin during pathological inflammation in autoimmune and non-autoimmune disease models of rheumatoid arthritis (RA), multiple sclerosis (MS), wound healing and infection. In order to explain the obtained results in our functional studies as well as some previous results on the functional roles of plasmin during different tissue remodeling processes, I propose that there is a functional correlation between absence of plasmin and an inability to activate complement.

The role of plasminogen during autoimmune collagen type II-induced arthritis (CIA) was studied first. The data revealed that whereas 83% of wild-type (plg+/+) mice developed CIA, none of the plasminogen-deficient (plg−/−) mice got arthritis within a 40-day period. When plg−/− mice were injected with a mixture of monoclonal antibodies against collagen type II they developed arthritis within a 5-day period, whereas no arthritis could be seen in plg−/− mice, although these mice had normal binding of antibody to the cartilage surface. These data suggest that plasmin plays an essential role in the step between antibody binding and inflammatory cell infiltration during CIA, probably during the step of complement activation. When plg−/− and plg+/+ mice were injected intra-articularly with collagen type II or 0.9% NaCl following CIA induction, plg−/− mice developed typical CIA, but the disease was less severe than in the plg+/+ mice and restricted to the injected joints. Sustained tissue necrosis was found only in the plg−/− mice after the local injection. When the antigen-induced arthritis (AIA) model was used, plg−/− mice developed a much more severe arthritis than the plg+/+ mice. These results indicate that different forms of pathogenesis exist for CIA and AIA, and further emphasize the importance of trauma in the induction of CIA in plg−/− mice.

We further investigated the role of plasmin in experimental autoimmune encephalomyelitis (EAE), which is an autoimmune disease model for MS. During a 2-month period, the severity, incidence, mean onset day, mean maximal score and mean accumulative score of EAE were essentially identical in plg−/− and plg+/+ mice of B10.Q background. Histopathological studies revealed similar levels of inflammation and demyelination in plg−/− and plg+/+ mice. These data indicate that plasmin does not play an essential role in the development of EAE. The findings that plasmin is essential for the development of CIA but not needed for the development of EAE suggest that plasmin may play a pivotal role in autoimmune diseases where complement activation is critically involved in the pathogenesis.

The role of plasmin was also studied in a tympanic membrane (TM) wound healing model. After TM perforations were performed, the plg+/+ TMs had all healed by day 11, whereas TM healing was completely arrested in plg−/− mice even as late as day 143. Immunohistochemical studies revealed a disturbed inflammation and tissue remodeling pattern in plg−/− mice. These data indicate that plasmin plays a central role in the healing of TM perforations.

The involvement of plasminogen in ear infections was also investigated in plg−/− mice. During an 18-week experimental period, spontaneous otitis media (OM) was essentially developed in all of the plg−/− mice, whereas all of the plg+/+ mice kept a normal TM status. Positive bacterial growth was found in 5 out of 6 plg−/− mice, but only in 1 out of 6 plg+/+ mice. Immunohistochemical studies showed an accumulation of inflammatory cells, fibrin and also other extracellular matrix in the middle-ear cavity and the external-ear canal of plg−/− mice. These results show a spontaneous development of OM in plg−/− mice, but not in plg+/+ controls, suggesting that plasmin plays a critical role in the defense mechanisms during ear infections.

Taken together, plasmin appears to play essential roles during autoimmune and non-autoimmune diseases in which complement activation is critical in the pathogenesis.

Keywords: Plasmin, complement, inflammation, wound healing, infection, autoimmune disease, non-autoimmune disease, rheumatoid arthritis, multiple sclerosis, tympanic membrane, otitis media.

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*, equal contribution.
INTRODUCTION

The extracellular matrix (ECM) is a dynamic and complex meshwork consisting of proteins such as collagen, fibronectin and proteoglycans (Price et al., 1997). The ECM is crucial for maintenance of the structural integrity of organisms. Cell-cell and cell-ECM interactions provide information essential for the control of cell morphogenesis, cell migration, tissue repair, and cell death. Degradation or activation of cell-surface and ECM proteins by proteolysis can mediate rapid and irreversible responses to changes in the cellular micro-environment. In many physiological and pathological processes that involve proteolytic degradation of the ECM, the plasminogen activator (PA) system has been suggested to play a key role in ECM remodeling (Mignatti and Rifkin, 1993).

The PA system is a versatile, temporally-controlled enzymatic system, the key component of which is the broad-spectrum protease plasmin. Plasmin is formed from plasminogen by either of two PAs, tissue-type PA (tPA) or urokinase-type PA (uPA). Activation of the PA system is initiated with the release of PAs by specific cells in response to external signals, and results in local extracellular proteolytic activity (Saksela and Rifkin, 1988; Vassalli et al., 1991). The PA system is also regulated by specific inhibitors, such as PA inhibitor type 1 (PAI-1) and PA inhibitor type 2 (PAI-2) which are directed against PAs, and α2-antiplasmin (α2-AP) which is directed against plasmin (Saksela and Rifkin, 1988; Ny et al., 1993).

THE AIMS OF THIS THESIS were to study the functional roles of plasmin during inflammation in autoimmune and non-autoimmune murine disease models. For this purpose, plasminogen-deficient mice were used. Initially, the role of plasminogen during autoimmune collagen type II-induced arthritis (CIA) was studied. By comparing the phenotypes of plasminogen-deficient mice between CIA and antigen-induced arthritis (AIA), the different roles of plasminogen in these two arthritis models were proposed. To further investigate the function of plasminogen in autoimmunity, experimental autoimmune encephalomyelitis (EAE), a mouse model for the autoimmune multiple sclerosis (MS) model, was induced in plasminogen-deficient mice. The role of plasminogen during non-autoimmune disease was studied in a tympanic membrane (TM) wound healing model. The involvement of plasminogen in host defense was also examined in plasminogen-deficient mice with spontaneous otitis media (OM). Based on the results obtained from my research and previous studies by other groups, I propose that there is a functional correlation between plasmin formation and complement activation. This hypothesis can explain some of the functional roles that plasmin seems to play in different tissue remodeling processes.

1. The extracellular matrix

There are four major types of tissue in animals: connective tissue, epithelial tissue, nerve tissue, and muscle. In connective tissue, the ECM plays a central role in maintaining and developing tissue architecture, homeostasis and in providing structural support in the body. In addition, the matrix influences basic cellular processes such as proliferation, differentiation, migration and adhesion (Matrisian, 1992). Two major forms of ECM can be identified, interstitial connective tissue and the basement membrane (Price et al., 1997).
1.1. The interstitial connective tissue

The interstitial connective tissue, which has a major supportive and mechanical function, is composed of connective tissue cells such as fibroblasts, osteoblasts, chondrocytes and macrophages, which are embedded in a complex meshwork of fibrous ECM. The major components of ECM are collagen types I, II and III, fibronectin, elastin, various proteoglycans and hyaluronan (Scott, 1995; Price et al., 1997). Variations in the relative amounts of the different types of matrix macromolecules and the way they are organized in the ECM give rise to a diversity of forms.

1.2. Basement membranes

Basement membranes are thin sheet-like structures that are “sandwiched” between an epithelial layer and the underlying connective tissue (Yurchenco and Schittny, 1990). The main components of the basement membrane are collagen type IV, laminin, entactin and different proteoglycans (Mosher et al., 1992). The basement membranes have several functions. They can serve as support for cells, function as molecular sieves and regulate cell differentiation and gene expression (Yurchenco and Schittny, 1990; Boudreau and Bissell, 1998).

1.3. Extracellular matrix remodeling

Degradation and remodeling of the ECM has been associated with many physiological and pathological processes such as embryo implantation, ovulation, wound healing and tumor invasion (Ny et al., 1993; Romer et al., 1996). Due to the complex composition of the ECM, different proteases with different substrate specificities are required for ECM degradation and remodeling. Among these proteases, the plasminogen activator (PA) system has been suggested to have important roles in ECM degradation and remodeling (Mignatti and Rifkin, 1993).

2. The plasminogen activator system

Plasmin is the key component of the PA system. It is a broad-spectrum protease which has the ability to degrade several components of the ECM including fibrin, gelatin, fibronectin, laminin and proteoglycans (Alexander CM and Werb, 1991). In addition, plasmin can convert some pro-matrix metalloproteinases (pro-MMPs) to active MMPs. It has therefore been suggested that plasmin may be an important upstream regulator of extracellular proteolysis (Werb et al., 1977; He et al., 1989). Plasmin is formed from the zymogen plasminogen through proteolytic cleavage by either of two physiological PAs, tPA or uPA (Saksela and Rifkin, 1988). As plasminogen is present in plasma and other body fluids at relatively high levels, the regulation of the PA system occurs mainly at the level of synthesis and activity of the PAs. Synthesis of the components of the PA system is highly regulated by different factors such as hormones, growth factors and cytokines (Saksela and Rifkin, 1988; Andreasen et al., 1997). In addition, there exist specific physiological inhibitors of plasmin and PAs. The main inhibitor of plasmin is α2-antiplasmin (Travis and Salvesen, 1983). The activity of PAs is regulated by PAI-1, which inhibits both uPA and tPA, and PAI-2, which inhibits mainly uPA (Thorsen et al., 1988; Belin, 1993; Binder et al., 2002). Certain cells also have a specific cell-surface receptor for uPA (uPAR) that can direct proteolytic activity to the cell surface (Stopelli et al., 1985; Vassalli et al., 1985). A simplified diagram of the PA system and its regulation is shown in Figure 1.
Figure 1. Schematic representation of the PA system and its regulation. The synthesis of tPA and uPA by specific cells is regulated by hormones, growth factors, and cytokines. In the extracellular space, the activities of PA and plasmin are controlled by the specific inhibitors PAI-1, PAI-2 and α2-AP. Binding of PAs and plasmin to cellular binding sites (R) can result in localized proteolytic activity on the cell surface.

2.1. Biochemical properties of plasminogen and plasmin

Plasminogen is a single-chain glycoprotein consisting of 790 amino acids with a molecular mass of approximately 92 kDa (Wiman and Wallen, 1975; Saksela and Rifkin, 1988). Plasminogen is mainly synthesized in the liver and is abundant in most extracellular fluids. In plasma the concentration of plasminogen is approximately 2 µM (Wallén P, 1980). Plasminogen therefore constitutes a large potential source of proteolytic activity in tissues and body fluids (Raum et al., 1980; Wallén P, 1980). Plasminogen exists in two molecular forms: Glu-plasminogen and Lys-plasminogen. The native secreted and uncleaved form has an amino-terminal (N-terminal) glutamic acid and is therefore designated Glu-plasminogen (Wallen and Wiman, 1970; Wallen and Wiman, 1972). However, in the presence of plasmin, Glu-plasminogen is cleaved at Lys76-Lys77 to become Lys-plasminogen (Wallen and Wiman, 1970; Wallen and Wiman, 1972; Wiman and Wallen, 1975). Compared to Glu-plasminogen, Lys-plasminogen has a higher affinity for fibrin and is activated by PAs at a higher rate (Bachmann F, 1987). These two forms of plasminogen can be cleaved at the Arg560-Val561 peptide bond by uPA or tPA, resulting in the formation of the disulphide-linked two-chain protease plasmin (Sottrup-Jensen et al., 1975). The amino-terminal part of plasminogen contains five homologous triple-loops, so-called kringles, and the carboxyl-terminal part contains the protease domain. Some of the kringles contain lysine-binding sites which mediate the specific interaction of plasminogen with fibrin and its inhibitor α2-AP (Wiman and Wallen, 1977; Wiman and Collen, 1979). A novel and interesting finding is that a 38-kDa fragment of plasminogen, consisting of kringles 1-4, is a potent inhibitor of
angiogenesis (O'Reilly et al., 1994). This fragment is termed angiostatin and can be generated from plasminogen through proteolytic cleavage by several MMPs (Dong et al., 1997; Patterson and Sang, 1997).

The main substrate for plasmin is fibrin, and dissolution of fibrin is pivotal for prevention of pathological blood clot formation (Collen and Lijnen, 1991). Plasmin also has substrate specificities for several other components of the ECM, including laminin, fibronectin, proteoglycans and gelatin, indicating that plasmin also plays an important role in ECM remodeling (Saksela and Rifkin, 1988; Alexander and Werb, 1989; Mignatti and Rifkin, 1993). Indirectly, plasmin can also degrade additional components of the ECM through its ability to convert some pro-MMPs to active MMPs, including MMP-1, MMP-2, MMP-3 and MMP-9 (Werb et al., 1977; He et al., 1989; Ginestra et al., 1997; Carmeliet et al., 1997a). It has therefore been suggested that plasmin may be an important upstream regulator of extracellular proteolysis (Collen, 2001). In addition, plasmin has the ability to activate latent forms of certain growth factors (Rifkin et al., 1990; Andreasen et al., 1997; Rifkin et al., 1999). In vitro, plasmin also cleaves components of the complement system and thereby release chemotactic complement fragments (Lachmann et al., 1982; Schaiff and Eisenberg, 1997).

2.2. Plasminogen activators

Two physiological PAs have been identified, uPA and tPA. Both PAs can activate plasminogen, but they are immunologically distinct molecules encoded by different genes (Dano et al., 1985). Although the homology between tPA and uPA at the amino acid level is only about 40%, the enzymes are highly similar in their basic structures (Saksela and Rifkin, 1988). They both have a protease domain at the carboxy-terminal end. The amino-terminal ends of uPA and tPA contain kringle domains (tPA contains two kringle domains, whereas uPA contains only one) and an epidermal growth factor (EGF)-like domain. In addition, tPA contains a finger domain that has a high-affinity binding site for fibrin. This finger domain is not present in uPA (Mignatti and Rifkin, 1993).

Expression of both uPA and tPA has been detected in a number of tissues and their synthesis is modulated by a variety of effector molecules such as peptide hormones, steroid hormones and growth factors (Saksela and Rifkin, 1988). Traditionally, different biological functions were suggested for the two PAs, tPA being primarily involved in vascular fibrinolysis and uPA mediating tissue remodeling processes (Mignatti and Rifkin, 1993). However, studies in PA-deficient mice suggest that tPA and uPA may have partially overlapping physiological functions (Carmeliet et al., 1994; Khokha et al., 1995; Carmeliet and Collen, 1996).

tPA is secreted as a 530-amino acid single-chain glycoprotein with a molecular mass of 68 kDa (Rijken and Collen, 1981; Pennica et al., 1983). Single-chain tPA can be converted into the disulfide-linked two-chain tPA by cleavage of the Arg^{275}-Ile^{276} bond by plasmin (Wallen et al., 1983). The carboxy-terminal (light chain) part of tPA contains the active site, while the non-catalytic amino-terminal (heavy chain) part of tPA contains structural domains, which is common for many blood proteins. Although two-chain tPA has higher proteolytic activity than single-chain tPA, both forms are active, and this property makes tPA unique among serine proteases (Rijken et al., 1982). In the absence of fibrin, tPA activates plasminogen rather slowly. However, in the presence of fibrin, tPA induced plasminogen activation is enhanced 200-400 times (Ranby
The binding of tPA to fibrin not only enhances plasminogen activation but also serves to localize plasmin to its substrate fibrin, thereby providing the targeted proteolysis which is an important characteristic of vascular fibrinolysis (Collen and Lijnen, 1991). Cellular binding sites for tPA have been described on different cell types, including hepatocytes and endothelial cells (Vassalli et al., 1991). However, a unique cell-surface receptor which exclusively binds tPA has, so far, not been identified (Hajjar et al., 1987; Verrall and Seeds, 1989).

uPA is secreted as a 411-amino acid single-chain glycoprotein with a molecular mass of 54 kDa (Nielsen et al., 1982; Wun et al., 1982). Single-chain uPA is an inactive pro-enzyme which is converted to an active disulfide-linked two-chain molecule through proteolytic cleavage of the Lys^{158}-Ile^{159} bond by enzymes such as plasmin, kallikrein, factor XIIa and cathepsin B (Ichinose et al., 1986; Andreasen et al., 1997). Unlike tPA, uPA has its own specific cell-surface receptor uPAR, which binds to and directs uPA activity to the cell surface (Romer et al., 1994; Solberg et al., 1994). This receptor has been identified on the surfaces of various cells (Blasi et al., 1986). uPAR is a 313-amino acid glycoprotein with a molecular weight of 55 kDa. It is inserted into the plasma membrane through a glycoprophospholipid anchor (Stoppelli et al., 1985; Vassalli et al., 1985; Blasi et al., 1986). The binding of uPA to uPAR seems to enhance plasmin formation on the cell surface (Plow et al., 1986). It has also been shown that uPAR is involved in the internalization of uPA/inhibitor complexes and also in cell migration and cell signaling events (Blasi, 1996; Andreasen et al., 1997).

2.3. Inhibitors of the plasminogen activator system

At the protein level, the PA system is strictly regulated by specific inhibitors directed against PAs and plasmin. These inhibitors include α₂-AP, PAI-1, PAI-2 and protease nexin 1 (PN-1). All of these inhibitors belong to the serine protease inhibitor (serpin) family (Irving et al., 2000; Gettins, 2002). Serpins are physiologically important molecules. They are involved in the control of all major proteolytic cascades in the body including fibrinolysis, coagulation, inflammation, carcinogenesis, apoptosis, angiogenesis and complement activation (Huber and Carrell, 1989; Gettins et al., 1993; Silverman et al., 2001). Members of this family are structurally related proteins with a similar tertiary structure, and are likely to have evolved from a common ancestor (Huber and Carrell, 1989). Serpins are so-called “suicide inhibitors” that mimic the substrate and trap the enzyme in an inactive stable 1:1 complex (Bode et al., 1994). Active serpins are metastable molecules and readily convert to more stable cleaved or latent forms when the cleaved or intact (respectively) reactive center loop becomes inserted into β-sheet A (Huber and Carrell, 1989). The metastability of the serpins is utilized in the inhibitory mechanism, where cleavage and insertion of the reactive centre loop results in a translocation of the covalently-bound protease to the opposite pole of the serpin molecule (Wilczynska et al., 1995; Huntington et al., 2000; Fa et al., 2000).

In addition to serpins, α₂-macroglobulin is a potent inhibitor of many proteinases, including MMPs and plasmin. The protein is synthesized in the liver and is present in plasma. α₂-macroglobulin displays a 30-amino acid bait region containing cleavage sites for proteases of all major classes (Travis and Salvesen, 1983; Sottrup-Jensen and Birkedal-Hansen, 1989). Hydrolysis anywhere within this region triggers α₂-macroglobulin to undergo conformational changes, resulting in a tight complex that strictly blocks the access of proteases to the substrate.
However, due to its large size (750 kDa), $\alpha_2$-macroglobulin has a limited ability to penetrate tissues, which restricts its sites of activity.

$\alpha_2$-antiplasmin ($\alpha_2$-AP) is the major physiological inhibitor of plasmin (Moroi and Aoki, 1976; Collen, 1976). $\alpha_2$-AP is a 452-amino acid single-chain glycoprotein with a molecular weight of 67 kDa (Wiman and Collen, 1977). Like plasminogen, the main site of $\alpha_2$-AP synthesis is the liver, and the concentration in plasma is about 70 $\mu$g/ml (Collen and Wiman, 1979). The interaction between $\alpha_2$-AP and plasmin is very fast and involves both the reactive center and the lysine binding sites of plasmin (Collen and Wiman, 1979; Sasaki et al., 1986). However, when plasmin is bound to its substrate fibrin, the lysine binding site is protected and the interaction with $\alpha_2$-AP is therefore much slower. In the circulation, this mechanism is thought to ensure that plasmin activity is restricted to fibrin (Longstaff and Gaffney, 1991).

PAI-1 efficiently inhibits single-chain tPA, two-chain tPA and two-chain uPA, and is therefore an important regulator of plasminogen activation (Berrettini et al., 1989). PAI-1 is a 50-kDa, 379-amino acid single-chain glycoprotein (Ny et al., 1986). PAI-1 is present in plasma and is also synthesized by many tissues and cells (Kristensen et al., 1990). In the blood and ECM, PAI-1 is mainly found in complex with vitronectin (Declerck et al., 1988). This binding stabilizes and maintains PAI-1 in its active conformation, but does not interfere with the inhibition of PAs (Seiffert et al., 1990). PAI-1 can exist in at least two different forms, an active and a latent form. In the absence of vitronectin, PAI-1 is rapidly converted from the active to the latent form. PAI-1 inhibits target proteolysis by rapidly forming covalently bound 1:1 complexes (Lawrence et al., 1989; Fa et al., 1995). It is thought that the primary function of PAI-1 in vivo is to balance the proteolytic activity of the PAs in fibrinolysis (Schleef and Loskutoff, 1988). PAI-1 is also involved in the regulation of cell adhesion, cell migration and invasion (Waltz et al., 1997; Kjoller et al., 1997).

PAI-2 is a 425-amino acid single-chain protein belonging to the ovalbumin branch of the serpin family (Schleuning et al., 1987; Ye et al., 1987). PAI-2 is a unique serpin, as it is the only wild-type serpin that polymerizes spontaneously under physiological conditions (Mikus et al., 1993; Mikus and Ny, 1996). PAI-2 exists in two molecular and topological forms, an intracellular non-glycosylated 46-kDa form and an extracellular glycosylated 60-kDa form (Genton et al., 1987). Although the biological function of extracellular PAI-2 remains speculative, it was thought that its primary function is to inhibit both uPA and two-chain tPA. However, PAI-2 is a poor inhibitor of single-chain tPA (Kruithof, 1988). Thus, extracellular PAI-2 may play an integral role in regulating uPA activity in complex processes including pregnancy, cancer formation and inflammation (Bachmann, 1995; Kruithof et al., 1995). The intracellular form of PAI-2 is known to bind other proteins, but its intracellular reactive protease is not yet known (Jensen et al., 1996; Darnell et al., 2003). Recent studies suggest that the CD-loop of PAI-2 functions as a redox-sensitive molecular switch that converts PAI-2 between a stable active monomeric conformation and polymerogenic conformation, suggesting that the redox status of the cell could be a regulator of PAI-2 polymerization (Wilczynska et al., 2003).

PN-1 is a 392-amino acid 45-kDa glycoprotein which is expressed in several tissues and cell types (Baker et al., 1980; Vassalli et al., 1993). PN-1 inhibits not only uPA but also plasmin, trypsin and thrombin quite efficiently (Scott et al., 1985). However, the physiological function of PN-1 is not well established.
2.4. Biological functions of the plasminogen activator system

The PA system has a well-established role in fibrinolysis (Nicoloso et al., 1988). In addition, the PA system has been suggested to play an important role as a source of proteolytic activity during many physiological and pathological processes such as tissue remodeling and cell migration, tumor invasion, angiogenesis, wound healing, ovulation, embryo implantation and arthritis (Mignatti and Rifkin, 1993; Khokha et al., 1995; Price et al., 1997; Ny et al., 2002). Our studies in plasminogen-deficient mice have suggested that plasmin may have more profound roles during different inflammatory disease processes, probably through functional correlation with the complement system (Papers I-V and Chapter 9 in this thesis).

Vascular fibrinolysis. The PA system is responsible for clearing the vascular system from fibrin clots (Collen, 1999; Rijken and Sakharov, 2001). This process is called vascular fibrinolysis (Ranby and Brandstrom, 1988; Angles-Cano, 1994). Although tPA and uPA are present in the plasma at similar concentrations, tPA is thought to be the functionally important PA in vascular fibrinolysis. The role of uPA in fibrinolysis is less well defined. Although uPA lacks affinity for fibrin, studies of tPA-deficient mice suggest that uPA may have a compensatory role in the fibrinolytic process (Carmeliet et al., 1994). Clinical studies of patients with deficiency in fibrinolytic activity have revealed the importance of a strict balance between PAs and PA inhibitors in vascular fibrinolysis (Schneiderman et al., 1991; Malmberg et al., 1994). Patients who lack or have reduced PAI-1 levels in plasma suffer from bleeding problems (Dieval et al., 1991; Lee et al., 1993), whereas inordinately high levels of PAI-1 are associated with myocardial infarction (Hamsten et al., 1985), deep venous thrombosis (Juhan-Vague et al., 1987), pulmonary embolism (Lang et al., 1998), diabetes (Gray et al., 1993), and arteriosclerosis (Glowinska et al., 2003).

Tumor invasion and metastasis. During tumor development, proteolytic degradation of the basement membrane ECM is required in order for tumor cells to invade surrounding tissue and to form metastasis (Terranova et al., 1986; Kleinman et al., 2001). Cancer metastasis is a result of several interdependent processes that include detachment of cancer cells from their original locations, cancer cell migration and invasion of the surrounding tissue, access of cancer cells to blood and lymphatic vessels, and adhesion to and invasion through the endothelium, allowing colonization at distant sites of the organism (Ahmad and Hart, 1997; Woodhouse et al., 1997; Fidler, 2002). In cooperation with the MMP system, the PA system has been suggested to provide the required proteolytic activity for these processes (Thorgeirsson et al., 1994; Stack et al., 1998). Of the components of the PA system, it is mainly uPA and its receptor uPAR that have been suggested to play a role in tumor formation and metastasis. This is based on two lines of evidence. Firstly, many animal model systems have provided evidence that uPA has a causal role in metastasis. Secondly, in many types of tumors, high levels of uPA and also uPAR and PAI-1, are correlated with poor patient prognosis (Andreasen et al., 1997). In addition, PAI-1 also seems to play an important role in several malignant processes during tumor development, such as tumor cell invasion, metastasis and neovascularization (Foekens et al., 2000).

Angiogenesis. The process of forming new capillaries from pre-existing vessels is called angiogenesis (Mignatti and Rifkin, 1996a). In order to initiate angiogenesis, the endothelial cells have to degrade basement membranes and interstitial stroma to facilitate migration into new
tissue (Molema et al., 1997; Heissig et al., 2003). Physiological angiogenesis is quite rare in the adult organism, and mainly takes place in corpus luteum formation and embryo development (Ribatti et al., 1991; Hazzard and Stouffer, 2000; Augustin, 2000). However, in the pathological process of tumor growth and metastasis, angiogenesis is an essential requirement (Juczewska and Chyczewski, 1997; Gupta and Qin, 2003). Several studies have indicated the involvement of PAs in this process (Pepper, 2001; Rakic et al., 2003). However, studies using PA- or plasminogen-deficient mice have shown that a PA-independent fibrinolytic pathway may exist and that the involvement of the PA system in angiogenesis may not be as important as anticipated (Carmeliet and Collen, 1996; Hiraoka et al., 1998).

**Ovulation.** In mammals, there is a continuous recruitment and development of follicles during the reproductive cycle. In order to release the oocyte from the pre-ovulatory follicle, the basement membrane and the connective tissue that surround the follicle must be loosened (Ny et al., 1993). The connective tissue contains several ECM proteins including type IV collagen, fibronectin, elastin and proteoglycans (Palotie et al., 1984). Many studies have suggested that the PA system has a functional role during ovulation. Plasminogen is present in follicular fluid and plasmin can partially degrade samples of follicular wall tissue in vitro (Beers et al., 1975). Furthermore, studies have shown that tPA, uPA and PAI-1 are present in the rat ovary and regulated by gonadotropins in a cell-specific and time-coordinated manner (Canipari and Strickland, 1985; Ny et al., 1985; Peng et al., 1993). These results suggest that a controlled and directed proteolytic activity generated by tPA and modulated by PAI-1 is involved in the rupture of selected follicles during ovulation.

However, extensive studies on protease-deficient mice have failed to show an essential role for tPA, uPA, PAI-1 and plasmin in ovulation (Leonardsson et al., 1995; Ny et al., 1999). The studies using the gonadotrophin-induced ovulation model reveal that ovulation efficiency is normal in singly deficient mice lacking either tPA, uPA or PAI-1. In tPA/uPA doubly deficient mice and in plasminogen-deficient mice, there is a small reduction in ovulation efficiency. These data suggest that under physiological conditions, plasmin is not necessary for follicular rupture or for the activation of other proteases that could be involved in ovulation (Ny et al., 1999).

**Embryo implantation.** During embryo implantation, trophoblast cells must invade the uterine epithelium by a process that requires proteolytic activity. It has been suggested that the PA system contributes to the proteolytic activity required for this process (Strickland and Richards, 1992). Despite much indirect evidence for a functional role of uPA during embryo implantation, mice deficient in uPA can apparently undergo a functional implantation, as these mice appear to have normal pregnancy and deliver litters with normal sizes (Carmeliet et al., 1994). The only phenotype related to implantation found in uPA-deficient mice is a reduced rate of trophoblast migration during early embryogenesis (Carmeliet and Collen, 1996).

**Brain function.** Indirect lines of evidence have suggested that the PA system may play a role in brain function. In rats, both tPA and uPA are expressed during neural development (Sumi et al., 1992), and tPA is induced as an immediate-early gene during seizure, kindling and long-term potentiation, suggesting that tPA may be involved in neural plasticity (Qian et al., 1993; Sappino et al., 1993). The involvement of the PA system in brain function will be discussed in more detail in Chapter 5.
2.5. Mice deficient in various components of the plasminogen activator system

As discussed above, the PA system has been associated with various tissue remodeling processes. The introduction of the gene targeting or 'knock-out' technology has allowed investigators to generate strains of mice that lack individual proteins. Studies of gene-deficient animals can confirm already known functions of these proteins in biological processes, and may also serve to reveal functional roles that have not been noted previously. Mice deficient in most of the components of the PA system, including tPA, uPA, uPAR, PAI-1, PAI-2, fibrinogen and plasminogen have been produced (Carmeliet et al., 1993a; Carmeliet et al., 1994; Ploplis et al., 1995; Bugge et al., 1995a; Bugge et al., 1995b; Bugge et al., 1996; Dewerchin et al., 1996; Dougherty et al., 1999). Surprisingly, all of these deficient mice survived embryonic development, were born with normal appearance and were fertile.

**tPA-deficient mice** have an impaired capacity for plasma clot lysis and an increased thrombotic tendency after injection of the pro-inflammatory thrombotic agent endotoxin (Carmeliet et al., 1994). These findings confirm that tPA has a role in vascular fibrinolysis. Further studies have indicated that tPA plays a role in neuronal processes including long-term potentiation (learning) and neuronal degeneration (Tsirka et al., 1995; Huang et al., 1996). Furthermore, tPA also seems to be important in protection against inflammatory renal injury (Kitching et al., 1997).

**uPA-deficient mice** are susceptible to pro-inflammatory thrombotic agents. This susceptibility seems to be caused by an impaired fibrinolytic function of macrophages, rather than reduced vascular fibrinolysis as in tPA-deficient mice (Carmeliet et al., 1994). Studies of uPA-deficient mice in many physiological processes that involve cell migration and invasion including ovulation, embryo implantation and development, imply a less important role for uPA than previously anticipated (Carmeliet et al., 1994; Leonardsson et al., 1995; Carmeliet and Collen, 1996). However, the apparently essential role of uPA in polyoma middle T-induced tumor invasion as well as in neointima formation after vessel-wall injury, suggests that uPA may be important for cell invasion and migration during other pathological processes such as inflammation, mild glomerulonephritis and arthritis (Carmeliet and Collen, 1996; Kitching et al., 1997; Sabapathy et al., 1997).

**tPA/uPA doubly deficient mice** have shorter life span, with growth retardation, a wasting syndrome, chronic ulceration, rectal prolapse and fibrin deposition in several organs including the lung, liver, kidney (Carmeliet et al., 1994). The induced phenotypes of tPA/uPA doubly deficient mice include impaired neointima formation, reduced fertility and ovulation, cachexia and severe glomerulonephritis (Carmeliet et al., 1994; Carmeliet and Collen, 1996; Kitching et al., 1997).

To date, no obvious phenotypes have been found in uPAR-deficient mice. This comes as a surprise, since uPAR has been suggested to play an important role in directing uPA activity in several processes (Bugge et al., 1995b; Dewerchin et al., 1996).

**PAI-1-deficient mice** have a reduced incidence of thrombotic, accelerated neointima formation, reduced lung inflammation and reduced arteriosclerosis, all of which confirm the role of PAI-1 in fibrinolysis (Carmeliet et al., 1993a; Carmeliet et al., 1993b; Carmeliet and Collen, 1996). It has also been reported that PAI-1-deficient mice have an accelerated rate of wound closure (Chan et al., 2001). Tumors in PAI-1-deficient mice displayed lower proliferative and higher apoptotic
indices and exhibited different neo-vascular morphology as compared to wild-type control mice (Gutierrez, 2000).

**PAI-2-deficient mice** have normal development, survival and fertility. No difference could be observed in response to a bacterial infectious challenge or endotoxin infusion. Monocyte recruitment was normal in these mice after thioglycollate injection. Epidermal wound healing was equivalent in the PAI-2-deficient and control mice (Dougherty et al., 1999).

### 2.6. Plasminogen-deficient mice

Plasminogen-deficient mice were generated by two research groups utilizing two separate strategies to inactivate the plasminogen gene (Ploplis et al., 1995; Bugge et al., 1995a). In mice, the plasminogen gene is located on chromosome 17 (Degen et al., 1990). One group eliminated exon 15, 16 and 17 (Ploplis et al., 1995) and the other replaced a 9-kb portion of the gene, resulting in a deletion of proximal promoter sequences as well as exon 1 and exon 2 (Bugge et al., 1995a). Surprisingly, the plasminogen-deficient mice were not embryonic-lethal but survived well into adulthood. Plasminogen-deficient mice had impaired thrombolysis and suffered from retarded growth, reduced fertility and life-span (Ploplis et al., 1995). No significant influence on hematological parameters was observed. This was surprising since mice deficient for both uPA and tPA suffered severe anemia (Carmeliet et al., 1994).

Spontaneous phenotypes of plasminogen-deficient mice include disturbed thrombosis and physical development. Fibrin deposition was found in a number of organs, with some associated pathological consequences. The occurrence of gastric and colonic ulcerations due to fibrin-mediated vasocclusion, with associated rectal ulceration, necrosis, inflammation and bacterial contamination, was a common phenotype in these mice. Reconstitution with murine plasminogen normalized the thrombolytic potential, indicating that plasminogen plays a critical role in dissolution of fibrin clots *in vivo* (Lijnen et al., 1996). Differences in physical development were observed only after 4 weeks of age, with less weight gain and delayed vaginal patency in plasminogen-deficient mice (Hoover-Plow et al., 1999). Ovulation efficiency in young, age-matched wild-type, plasminogen-heterozygous, and plasminogen-deficient female mice was studied after stimulation with gonadotrophin. These studies indicated a slight, but not statistically significant, reduction in ovulation efficiency (Ny et al., 1999). However, studies have demonstrated a critical, dose-dependent requirement for plasminogen in lactational differentiation and mammary gland remodeling during involution (Lund et al., 2000). Primiparous plasminogen-deficient mice have seriously compromised mammary gland development and involution. All mammary glands were under-developed and one-quarter of the mice even failed to lactate. Histological abnormalities of mammary glands were also observed in plasminogen-deficient mice (Lund et al., 2000).

Induced phenotypes of plasminogen-deficient mice include inflammation and infection, vascular remodeling, wound healing, neurodegeneration, rheumatoid arthritis (RA), and also cancer growth and metastasis. Some of these studies are summarized in Table 1. Most of them indicate a reduced recruitment of inflammatory cells and disturbed tissue remodeling processes, where a strong relationship between plasminogen and fibrin(ogen) has been suggested to contribute to these processes (Ploplis et al., 1995). Additionally, other fibrin(ogen)-independent mechanisms have been revealed from studies with plasminogen/fibrinogen doubly deficient mice and indicate that
the substrate specificity of plasmin in vivo is more diverse. For instance, necrosis has been found in both plasminogen-deficient and plasminogen/fibrinogen doubly deficient mice during many pathological models, suggesting there exist a fibrin-irrelevant pathway through which plasmin could play a critical role. I have recently proposed that there may be a functional correlation between the PA and complement systems during tissue remodeling processes. This hypothesis, as will be discussed in more detail in Chapter 9, provides a novel angle to explain the possible underlying mechanism for these results that can not be explained by the fibrinolytic function of plasmin. The conflicting results obtained from plasminogen-deficient mice in different models may also be the result of different methods used to challenge the hosts as well as the diverse mechanisms initiated after injury during the host reaction process (For references, see Table 1).

2.7. Human plasminogen deficiency disorders

The human plasminogen gene spans about 52.5 kb of DNA and consists of 19 exons separated by 18 introns (Petersen et al., 1990). The plasminogen gene is located on chromosome 6 at position 6q26 (Murray et al., 1987).

The first documented patient with abnormal plasminogen was reported in 1978, who suffered from a history of thrombotic occurrences (Aoki et al., 1978). Although the plasminogen antigen concentration was normal, the patient only had 37% functional plasminogen activity. Further studies revealed an active site defect due to a G→A transition in exon XV of the plasminogen gene which caused an Ala601→Thr substitution near His603 of the active site (Miyata et al., 1982; Ichinose et al., 1991). The Ala601→Thr substitution appears at a high frequency in the Japanese population (2%) as well as in other populations, and can therefore be used as a genetic marker (Nishimukai et al., 1981; Dykes et al., 1984).

Two types of plasminogen deficiency have been recognized (Ichinose et al., 1991). Patients who had a concordant deficiency in plasminogen activity and antigen were designated as having a type I deficiency, whereas patients who had decreased activity but a normal antigen level were designated as having a type II deficiency (Ichinose et al., 1991).

Dysfunctional plasminogen variants have also been described by several other investigators (Kazama et al., 1981; Wohl et al., 1982; Miyata et al., 1984). In many of these cases, the plasminogen variant has been associated with recurrent thrombosis. It is clear that both type I and type II plasminogen deficiencies predispose the individual to venous thrombosis, although most of the cases reported hitherto have been of type II, e.g., plasminogen Tochigi disease. Such mutations include Ala601→Thr, Val355→Phe and Ser572→Pro substitutions (Aoki et al., 1978; Ichinose et al., 1991; Azuma et al., 1993).

Another disease associated with plasminogen deficiency is ligneous conjunctivitis. Ligneous conjunctivitis is a rare and unusual form of chronic pseudo-membranous conjunctivitis which usually starts in early infancy (Schuster and Seregard, 2003). The disease also includes pseudo-membranous lesions of other mucous membranes such as the mouth, nasopharynx, trachea and female genital tract. By examining two unrelated Turkish girls suffering from ligneous conjunctivitis and occlusive hydrocephalus, Schuster and his colleagues (Schuster et al., 1997) found an Arg216→His substitution in plasminogen gene of one girl, due to a homozygous G-to-A point mutation at nucleotide position 780. Her healthy sister and parents were heterozygous for
Table 1. Induced phenotypes of plasminogen-deficient mice.

<table>
<thead>
<tr>
<th>Types of challenge</th>
<th>Phenotypes</th>
<th>References</th>
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<tbody>
<tr>
<td><strong>Inflammation and infection</strong></td>
<td>Compromised macrophage and lymphocyte recruitment</td>
<td>(Ploplis et al., 1998)</td>
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<td></td>
<td>Decreased spirochete load of <em>Borrelia</em> fever</td>
<td>(Gebbia et al., 1999)</td>
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<td></td>
<td>Resistance to <em>Yersinia pestis</em> infection</td>
<td>(Goguen et al., 2000)</td>
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<td></td>
<td>Severe functional and histological exacerbation of glomerular injury after glomerulonephritis</td>
<td>(Kitching et al., 1997)</td>
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<td></td>
<td>Enhanced collagen-fibrin deposition and diminished macrophage recruitment after bleomycin challenge</td>
<td>(Swaisgood et al., 2000)</td>
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<td></td>
<td>Decreased spirochete load of <em>Borrelia</em> fever</td>
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<td>Enhanced collagen-fibrin deposition and diminished macrophage recruitment after bleomycin challenge</td>
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<td><strong>Vascular remodeling</strong></td>
<td>Impaired vascular wound healing and decreased arterial neointima formation</td>
<td>(Carrel et al., 1997b)</td>
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<td></td>
<td>Diminished inflammation, retarded lamina degradation, suppressed smooth muscle cell proliferation and migration</td>
<td>(Moons et al., 1998)</td>
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<td></td>
<td>Inability of leukocytes to penetrate neointima of the venous patch</td>
<td>(Shi et al., 1999)</td>
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<td></td>
<td>Accelerated lesion development in the proximal and distal aorta</td>
<td>(Xiao et al., 1997)</td>
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<tr>
<td></td>
<td>Defective arterial remodeling and adventitious collagen deposition, impaired prevention of medial atrophy in challenged vessels</td>
<td>(Drew et al., 2000b)</td>
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<td>Inhibited increase in adventitious areas of arteries, a severe alteration in intramural thrombus clearance</td>
<td>(Busuttil et al., 2000)</td>
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<td><strong>Wound healing</strong></td>
<td>Impaired keratinocyte migration; cell migration and tissue remodeling are not compromised in general</td>
<td>(Romer et al., 1996)</td>
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<td></td>
<td>Persistent fibrin deposition post laser photo keratectomy; Re-epithelialization was not compromised</td>
<td>(Drew et al., 2000a)</td>
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<td></td>
<td>Impaired healing process after blade injury with severe inflammatory response, unresolved scar tissue and fibrin deposition</td>
<td>(Kao et al., 1998)</td>
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<td></td>
<td>Persistent damage to centrilobular hepatocytes, impaired ability to degrade necrotic tissue</td>
<td>(Bezerra et al., 1999)</td>
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<td></td>
<td>Persistent existence of necrotic cardiomyocytes, defects of forming granulation tissue and fibrous tissue</td>
<td>(Creemers et al., 2000)</td>
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<td><strong>Neurodegeneration</strong></td>
<td>A resistance to excitotoxin-mediated hippocampal neuronal death, through tPA/plasminogen catalyzed disruption of neuron-laminin interactions</td>
<td>(Tsirka et al., 1997a; Tsirka et al., 1997b)</td>
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<td>Exacerbated axonal demyelination and an increase in fibrin deposition after sciatic nerve injury</td>
<td>(Akassoglou et al., 2000)</td>
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<td></td>
<td>Significantly larger size of focal cerebral infarct and an increase in fibrin deposition</td>
<td>(Nagai et al., 1999)</td>
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<tr>
<td><strong>Cancer and metastasis</strong></td>
<td>Primary lung carcinoma tumors were smaller, less hemorrhagic and skin ulceration were reduced; no quantitative differences in lung metastasis.</td>
<td>(Bugge et al., 1997)</td>
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<td></td>
<td>Adenocarcinomas developed normally; significantly reduced lung metastasis</td>
<td>(Bugge et al., 1998)</td>
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<tr>
<td><strong>Rheumatoid arthritis</strong></td>
<td>More fibrin deposition exacerbated joint inflammation</td>
<td>(Busso et al., 1998)</td>
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this mutation. The second patient showed homozygosity for a G-to-A point mutation at nucleotide position 1924, which led to a stop codon ($\text{Trp}^{597} \rightarrow \text{Ter}$) in plasminogen gene. In the child of a consanguineous Turkish couple, Schott and his colleagues found ligneous conjunctivitis and homozygous plasminogen deficiency due to homozygosity for a transversion, leading to a stop codon at position 460 ($\text{Glu}^{460} \rightarrow \text{Ter}$). Replacement therapy with Lys-plasminogen led to rapid regression of the pseudo-membranes and normalization of respiratory tract secretions and wound healing (Schott et al., 1998).

In our studies on ear infections in wild-type and plasminogen-deficient mice, we observed that virtually all the plasminogen-deficient mice gradually and spontaneously developed OM (Paper V). The spontaneous OM was diminished within days following supplementation of plasminogen, indicating that lack of plasminogen is the direct cause of the disease (data not shown). Considering that both the conjunctiva and the middle-ear cavity are highly sensitive mucous organs, it is reasonable to speculate that a chronic OM in humans could be associated with plasminogen abnormalities. Indeed, our studies have shown that members of at least one family suffering from chronic OM have drastically reduced plasminogen levels (unpublished data). Detailed investigations at the genome and protein levels are in progress. A genetic study has also been initiated to determine the incidence of low-plasminogen-related recurrent OM.

3. Inflammation

Inflammation is a localized protective response that serves to destroy, dilute or wall-off both the initial cause of cell injury and the consequences of such injury, the necrotic cells and tissues. Inflammation occurs in an attempt by leukocytes to defend the host from injury. The accumulation and subsequent activation of leukocytes are central events in the pathogenesis of virtually all forms of inflammation. Inflammation can play both a beneficial and a deleterious role. Absence of inflammation leads to a compromised host, resulting in uncontrolled infections, unhealed wounds and permanent festering sores in the injured organs. However, inflammation and the consequent tissue repair, either secondary to abnormal recognition of host tissue as ‘foreign’ or abnormal turn-off of an otherwise normal inflammatory process, may be potentially harmful, resulting in various disease, from life-threatening hypersensitivity reactions to common chronic diseases such as RA and MS (Sharma, 1992; Bruck and Stadelmann, 2003).

Regardless of the etiology, inflammation can generally be divided into acute and chronic forms. Both forms are amplified as well as propagated as a result of the recruitment of humoral and cellular components of the immune system (Kay, 1987; McCartney-Francis et al., 2003). Acute inflammation is characterized by the enhanced blood flow, increased vasopermeability with edema, induction of microhemorrhage and microthrombosis, and infiltration of the connective tissue by leukocytes, predominantly neutrophils (Ryan and Majno, 1977; Movat, 1987). At the site of injury, neutrophils or monocytes destroy offending agents, kill bacteria and other microbes and degrade necrotic tissue and foreign antigens (Movat et al., 1987; Cybulsky et al., 1988). Under certain circumstances, leukocytes may also prolong inflammation and induce tissue damage by releasing enzymes, chemical mediators and toxic oxygen radicals.

Chronic inflammation is characterized by the simultaneous occurrence of active inflammation, tissue destruction and attempts at healing (Davies and Allison, 1976; Majno, 1998; McCartney-Francis et al., 2003). In contrast to acute inflammation, chronic inflammation is characterized by
infiltration of mononuclear cells including macrophages, lymphocytes and plasma cells, as a reflection of a persistent reaction to injury (Haskill et al., 1992; Yamamoto, 2000). The inflammatory cells cause further tissue destruction (McCartney-Francis et al., 2003), and the body attempts to repair the injury by connective tissue replacement including angiogenesis, fibrosis, formation and remodeling of the ECM (Shaw, 1991; Jackson et al., 1997).

The vascular and cellular responses of both acute and chronic inflammation are mediated by chemical factors. Such mediators are derived from plasma or cells and triggered by the inflammatory stimulus, necrotic cells or tissues (Streetz et al., 2001; Villolslada and Genain, 2004). Once activated, these mediators amplify the inflammatory response and modify its evolution (Kollias et al., 1999). Inflammation is terminated when the injurious stimulus has been removed and the mediators have either been dissipated or inhibited. Morphological patterns in acute and chronic inflammation include serious inflammation, fibrinous inflammation, suppurative or purulent inflammation, and ulcer inflammation. Systemic effects of inflammation include fever, acute-phase reaction and a variety of changes of peripheral blood leukocytes (Nieman et al., 1999; Steiner and Branco, 2003). IL-1, IL-6, and TNF are important mediators of these reactions (Murch, 1998; Catania et al., 1999). During bacterial infection, elevation of leukocytes, in particular neutrophils and lymphocytes, caused by IL-1, TNF and/or CSF is a common feature of inflammatory reactions.

Inflammation plays a central role in the pathogenesis of autoimmune diseases, and also of non-autoimmune diseases such as infection and wound healing. Further understanding and better control of this complex inflammatory system offers a great challenge and opportunity for medical research. The PA system has been suggested to be involved in several aspects of the inflammatory response. In this thesis, I have attempted to investigate the functional roles of plasmin during different inflammatory diseases. The following chapters summarize our current understanding of these diseases, as well as my findings regarding the involvement of the PA system during these processes. Based on these findings, I propose that there is a functional correlation between absence of plasmin and an inability to activate complement, as will be described in Chapter 9. This hypothesis is an attempt to explain the functional roles of plasmin during these tissue remodeling processes that can not be explained by the traditional theories about the functional roles of plasmin.

4. Rheumatoid arthritis (RA)

RA is a systemic chronic relapsing disease affecting 1-2% of human population. The incidence is approximately 2.5 times higher in women than in men (Lawrence et al., 1998), and the prevalence increases with age in both sexes. It has been reported that Caucasians are at a higher risk of developing RA than Asians and Africans (Oen and Cheang, 1996). The etiology of RA is complicated. The heritability of RA has been estimated to be between 53 and 65% (MacGregor et al., 2000). An association between RA and the HLA-DR4 and HLA-DR1 molecules have been found (Nepom et al., 1986; Lang et al., 1990). However, other studies have also indicated that non-genetic and environmental factors are important (Callahan, 2003).

The disease starts with an immunological attack against cartilage in the joints, resulting in synovitis, hyperplasia and angiogenesis (Paleolog, 2002). The disease generally progresses in relapses with substantial infiltrations of a newly-formed stromal tissue called pannus to the joint
space. This chronic inflammation can eventually result in joint deformity and loss of movement ability due to the complete destruction of cartilage and bone erosion (Lee and Weinblatt, 2001; Gravallese, 2002). RA may also have systemic involvement with autoantibody production and effects on internal organs. The exact mechanism of tissue destruction during RA remains unclear. However, several studies have suggested that the PA system (Saxne et al., 1993; Belcher et al., 1996; Busso et al., 1997) and the MMP system (Cawston, 1998; Keyszer et al., 1999) are involved. The diagnosis of RA is based on clinical criteria defined by the American Rheumatism Association (Arnett et al., 1988).

4.1. Components of joint and cartilage

Cartilage is composed of chondrocytes and the ECM. Main components of the ECM in articular cartilage are aggrecan and collagen type II. Aggrecan is a proteoglycan responsible for the shock-absorbing capacity of the joint cartilage. Collagen type II is a fibrillar protein composed of three identical chains which form a triple helix (Cremer et al., 1998). Collagen type II is mainly present in articular cartilage, but the presence of collagen type II in the middle and inner ear has also been shown (Yoo and Tomoda, 1988; Slepecky et al., 1992). Other proteins present in the cartilage include collagen IX and XI, and cartilage oligomeric matrix protein.

4.2. Inflammatory cells and molecules in rheumatoid arthritis

Many cellular and non-cellular members of the inflammation system have been suggested to play a role in RA. The synovial membrane encapsulating the joint consists of synoviocytes of type A (macrophages) and type B (fibroblasts). Normally, the macrophages in the lining layer of the synovial membrane degrade the debris from the joints and protect the joints from microbes.

During inflammation, the macrophages participate in the pathogenesis of the disease by producing not only the pro-inflammatory cytokines IL-1 and TNF-α, but also other cytokines such as IL-6, IL-12, IL-15, IL-18 and GM-CSF (Feldmann et al., 1996; Carteron, 2000; Arend, 2001). However, macrophages also produce the MMPs that cause the destruction of joint cartilage (Katrib et al., 2001). Massive infiltration of neutrophils was observed in the synovial fluid of acute RA patients, while macrophages were present in abundance in the synovial tissue (Arend, 2001). The neutrophils have an important role in the degeneration of the cartilage by secreting IL-1, superoxides, and other degrading enzymes (Edwards and Hallett, 1997). They also secrete chemokines and have an up-regulation of Fc receptor, which can bind antibodies directly (Felzmann et al., 1991).

Although high numbers of T cells and antigen-presenting cells have been found in the synovial tissue of RA patients (Sekine et al., 1999), there were arguments against the importance of T cells in RA pathogenesis; for instance, the depletion experiments for T cells were not efficient (Choy et al., 1992). Thus, the role of T cells in the pathogenesis of RA is still under debate (Fox, 1997).

The role of B cells in RA could either be as an antigen-presenting cell or a producer of auto-reactive antibodies. A recent report has shown that anti-B cell antibody treatment in RA patients significantly decreased the severity of the disease (Edwards and Cambridge, 2001). In the inflamed joints, complement/Ig complexes have been found along with membrane attack complex formation, implicating the involvement of complement components in RA pathogenesis.
Sanders et al., 1986). At early stages of RA, fibroblasts, macrophages, activated T and B cells, cytokines and the complement system work in concert to form the pannus tissue and mediate sustained inflammation.

4.3. Collagen type II-induced arthritis (CIA)

The most commonly used animal model for studies of human RA is the CIA model (Holmdahl et al., 2002). The cartilage collagen has long been focused as the potential auto-antigen in RA. CIA was first described in the rat, where collagen type II emulsified in complete Freund’s adjuvant (CFA) was used (Trentham et al., 1977). The triple-helical structure of native collagen type II is essential for disease induction, as denatured collagen type II could not induce arthritis in mice (Stuart et al., 1982). The susceptibility of CIA is strongly associated with major histocompatibility complex class II (MHC II) molecules (Holmdahl et al., 1988a; Gustafsson et al., 1990; Kjellen et al., 1998). In mice, H-2q and r bearing haplotypes are the most susceptible (Holmdahl et al., 1988a). Interestingly, transgenic mice expressing human HLA-DR1 and HLA-DR4 are susceptible to arthritis (Holmdahl et al., 1988a; Rosloniec et al., 1997; Andersson et al., 1998).

CIA has several similarities to human RA. Deposition of fibrin, immunoglobulin and complement, mass infiltration of neutrophils to the joint space, hyperplasia of synovial cells, pannus formation as well as marginal erosion of subchondral bone and cartilage can be seen in both RA and CIA (Trentham et al., 1977; Caulfield et al., 1982). However, a few dissimilarities between RA and CIA (induced with heterologous collagen in rats) also exist. For example, CIA is induced whereas RA occurs spontaneously, and rheumatoid factor cannot be found in CIA (Morgan et al., 1980). In addition, CIA is not self-perpetuating, and fluctuating remissions and exacerbations do not occur spontaneously as they do in human RA (Trentham et al., 1977).

Macrophages and neutrophils have an important role in the pathogenesis of CIA. Monomeric IgG and immune complexes in the joint can bind to Fcγ receptors expressed on macrophages and neutrophils. This can lead to cross-linking which triggers several effector functions such as release of inflammatory mediators by activated neutrophils (Huizinga et al., 1989), increase the capacity of antigen-presenting cells (Heijnen et al., 1996) and phagocytosis (Simms et al., 1991). TNF-α and IL-1 are the two main cytokines that have important roles in the pathogenesis of CIA (Stasiuk et al., 1996; Mussener et al., 1997). TNF-α plays a role in stimulating the inflammation but the destruction of the joint is more dependent on IL-1 (Hom et al., 1988; Joosten et al., 1999).

Both B and T cells are involved in CIA (Holmdahl et al., 1989a; Durie et al., 1994). After challenge with collagen type II, B cells start to produce anti-collagen type II antibodies, which are often highly cross-reactive to autologous collagen type II (Holmdahl et al., 1989b). Several depletion experiments in B cells have indicated that these cells are critical for the development of CIA (Helfgott et al., 1984; Svensson et al., 1998). Although the presence of T cells during CIA implicates the importance of T cells, transfer of T cells specific to collagen type II induces a sub-clinical disease (Holmdahl et al., 1986). The decrease of T cell numbers after onset of CIA suggests a more regulatory role, rather than a direct role, of T cells in the effector phase (Holmdahl et al., 1988b).
Complement activation has been suggested to play an important role during CIA (Terato et al., 1992; Yamamoto et al., 1996). In one study the role of C5 was investigated using C5-deficient and C5 proficient mouse strains. All of the four non-responder strains used in the study were C5-deficient, whereas all the strains that showed some degree of responsiveness were C5 proficient, indicating the necessity of C5 for the development of the disease (Ji et al., 2001). Systemic administration of an anti-C5 antibody effectively inhibited terminal complement activation in vivo and prevented the onset of CIA in immunized animals. Treatment with anti-C5 monoclonal antibodies was also highly effective in ameliorating established disease (Wang et al., 1995). Moreover, C5-deficient mice were completely resistant to CIA (Wang et al., 2000). Recently, an extensive study on the role of different members of the complement system in one arthritis model showed that the alternative pathway of complement activation is critical, while classical pathway components are entirely dispensable (Ji et al., 2002).

4.4. **Collagen type II antibody-induced arthritis (CAIA)**

Transfer of monoclonal or polyclonal antibodies directed against collagen type II to mice induces an acute non-erosive arthritis, so-called collagen type II antibody-induced arthritis (CAIA) (Holmdahl et al., 1990; Terato et al., 1992; Nandakumar et al., 2003). The transfer of anti-collagen type II antibodies seems to be independent of MHC, since both resistant and susceptible strains develop arthritis after transfer (Stuart et al., 1983; Stuart and Dixon, 1983). This model is an excellent tool for studies on the effector phase of the arthritis. By using this model, it is possible to distinguish the immune response from inflammation and tissue destruction. The mechanism of CAIA probably involves a binding of anti-collagen type II IgG to cartilage-specific antigens in the joint, which then activates the complement system via C1q, and/or direct binding to Fc receptor-expressing phagocytes. Consequently, this activation leads to a release of chemotactic factors which in turn attracts more neutrophils and macrophages to the site of inflammation. The activated phagocytes then release different proteases and reactive radicals which eventually damage and destroy the cartilage and bone. The main histopathology of CAIA is the swelling of the synovial tissue and infiltration of inflammatory cells, but only marginal erosion of cartilage and bone can be observed (Nandakumar et al., 2003).

4.5. **Antigen-induced arthritis (AIA)**

AIA was introduced at around the same time as CIA (Brackertz et al., 1977a; Brackertz et al., 1977b; Brackertz et al., 1977c). In this model, arthritis is induced by a single intra-articular injection of a protein antigen into the knee joint cavity of animals that have been pre-immunized with the same antigen emulsified in CFA. In mice and rats, the model is often induced by using a cationic antigen, usually methylated bovine serum albumin (mBSA) (Griffiths, 1992). Because of the methylation, mBSA has a positive net charge and is therefore bound and retained in the negatively-charged cartilage after intra-articular injection. Thereafter, the immune system further attacks the bound mBSA and induces inflammation and tissue destruction. The retention of the antigen in the joints is considered to be important for the chronicity of the inflammation. Unlike CIA, AIA is a monoarticular arthritis affecting only injected joints. Another feature of AIA is that the susceptibility is not associated with MHC class II.

The chronic phase of AIA is characterized by hyperplasia of the synovial lining layer, predominant infiltration of mononuclear cells into the synovium, and by cartilage destruction, i.e.
depletion of proteoglycans, necrosis of chondrocytes and formation of synovial pannus which erodes and destroys the cartilage and subchondral bone. The arthritis persists in this chronic state for weeks or months.

The pathogenic mechanisms responsible for the chronification of joint inflammation and associated cartilage destruction are not yet clear. A prerequisite is the retention of the antigen within the joint structures (Cooke et al., 1972; Cooke and Jasin, 1972), and a well-developed cell-mediated immunity directed against the specific antigen seems to be required (Brackertz et al., 1977c; Pettipher and Henderson, 1988). T cells have been suggested to play a critical role in the development of AIA. It has also been suggested that autoimmunity to cartilage components plays a role in the persistence of the joint inflammation and cartilage destruction (Brauer et al., 1993).

4.6. Role of the plasminogen activator system in arthritis

The PA system has been suggested to play important roles in inflammatory cell infiltration, fibrin deposition, and joint destruction associated with RA (Busso et al., 1997; Busso and Hamilton, 2002). In synovial tissues and fluids from RA patients, levels of tPA were reduced or unchanged, while uPA activity was increased. Increased levels of uPAR, PAI-1 and PAI-2 were also found in synovial tissues from RA patients (Brommer et al., 1992; Busso et al., 1997). Most cells that are present in the inflamed joint express uPA, together with variable amounts uPAR and PAIs. The increased uPA levels and decreased tPA expression found in synovial tissues and fluids from RA patients can probably be accounted for by the opposing effects of cytokines on the two PA genes (Busso and So, 1997). Secreted pro-uPA could induce plasmin-independent effects such as mitogenic, migratory and adhesiveness responses. Alternatively, active uPA could generate plasmin and thereby further activate and mobilize latent forms of growth factors that can influence the growth and differentiation of cellular constituents in arthritic joints. A uPA-mediated plasminogen activation seems to be involved in joint destruction and intra-articular fibrin turnover.

Joint damage with cartilage and bone destruction is believed to be mediated mainly through activation of MMPs and plasmin by activated synovial cells of the pannus, chondrocytes and inflammatory cells (Werb Z and Alexander CM, 1993). Plasmin may play a pivotal role in this degradative process, since it can directly degrade cartilage proteoglycans as well as other cartilage and bone matrix proteins. Plasmin may also act indirectly, through the activation of latent MMPs (Ronday et al., 1997). The complement system has also been shown to play an important role during development of RA. In vitro studies suggest that activation of the complement system may be plasmin-mediated (Kaplan et al., 1981; Kramer et al., 1992).

Fibrin deposition, a common feature of RA, is caused by a disturbed intra-articular balance between coagulation and fibrinolysis. Accumulation of intra-articular fibrin occurs on the synovial membrane, the cartilage surface and as particulate aggregates in synovial fluid (Weinberg et al., 1991). Fibrin deposits can have potential adverse effects; for example, the impairment of normal nutrition to these tissues, leading to hypoxia and acidosis in synovial fluid (Mapp et al., 1995). Fibrin(ogen) degradation products may also have a role in inflammation by increasing vascular permeability (Dvorak et al., 1985). Moreover, the fibrin meshwork serves as a provisional matrix to which inflammatory cells can adhere and migrate through both integrin and non-integrin receptors (Altieri, 1993; Forsyth et al., 2001). This potential arthrogenic role of
fibrin in RA is supported by studies on animals and reports from patients (Gordon and Bullough, 1982).

Studies of AIA in mice deficient in uPA, plasminogen and PAI-1 have indicated significantly increased levels of fibrin in joints of uPA or plasminogen-deficient mice, whereas lower levels of fibrin were detected in PAI-1-deficient mice (Busso et al., 1998; Van Ness et al., 2002). When mice were systemically treated with IL-1 after AIA induction, tPA-deficient mice had particularly severe disease. Fibrin deposition appeared to parallel disease severity under the various conditions, suggesting that PA-mediated fibrinolysis may play a protective role in AIA (Yang et al., 2001).

However, intra-articular injections of uPA revealed that uPA is an essential mediator of joint inflammation, possibly by stimulating pro-inflammatory cytokines IL-6, IL-1β and TNF-α (Jin et al., 2003). In a chronic systemic CIA model, it has been found that uPA mRNA levels increase during development of CIA (Busso and Hamilton, 2002). uPA-deficient mice with C57BL/6 background had only mild disease, suggesting a pro-inflammatory role for uPA (Cook et al., 2002). The reduced levels of IL-1β and TNF-α in the joints of uPA-deficient mice undergoing CIA may be caused by an inability to amplify cytokine levels in these mice. Furthermore, T cells from uPA-deficient mice produced less of the Th1 cytokine IFN-γ than T cells from wild-type controls, which is consistent with a previous report (Gyetko et al., 1999). In our study where a mixed C57BL/6 and DBA/1 background was used, we also observed less severity and less incidence of CIA in uPA-deficient mice after induction of CIA (Paper I). Thus, it appears that uPA can be deleterious or beneficial, depending on the animal model used. These findings highlight the complex nature and the different roles the same mediator can have in the pathogenesis of AIA and CIA.

tPA-deficient mice with a C57BL/6 genetic background were also found to develop a more severe CIA with greater accumulation of fibrin in the joints than wild-type mice (Cook et al., 2002). Similar results were obtained by using a mBSA/IL-1 mono-articular arthritis model (Yang et al., 2001). This suggests that tPA, but not uPA, may have an important role in fibrinolysis during CIA. However, the mice used in this study were of C57BL/6 (H-2b) strain, which is traditionally regarded as one of the least responsive strains (Wooley, 1988; Holmdahl et al., 1988a). In our studies on mice with a mixed C57BL/6 and DBA/1 background, tPA-deficient mice had similar severity and incidence of CIA as wild-type controls (unpublished data). The different roles of tPA during CIA in the different studies may reflect the complex genetic influence involved in CIA. More studies are required to further investigate the exact pathological role of tPA during CIA.

The relative importance and the contrasting roles of the PA system in the pathogenesis of RA are complicated, and reflect the complex nature of RA. Overall, the ultimate elucidation of the in vivo role of these molecules in the pathogenesis of arthritis could facilitate the development of new therapeutic strategies for RA.

5. Multiple sclerosis (MS)

MS is an autoimmune neurological disease that affects 0.1%-0.2% of the Caucasian population (MacDonald et al., 2000; Pozzilli et al., 2002). The disease often starts between 15 and 50 years
of age and is 3 times as frequent in women than in men (Benveniste, 1997). The symptoms of MS start with a feeling of weakness in the legs and progress towards motor disturbances. In severe cases, it can lead to paralysis and ultimately death. MS generally presents with relapses, but can also progress continuously (Lublin and Reingold, 1996).

MS is a demyelinating disease in which the immune system attacks the myelin sheets surrounding nerve cells in the central nervous system (CNS), followed by infiltrations of mononuclear cell and demyelinating lesions (Steinman, 1996). The axons are usually unaffected, however. Myelin is composed of different proteins such as myelin basic protein (MBP), proteolipid protein, myelin-associated glycoprotein and myelin oligodendrocyte glycoprotein (MOG).

Several proteins have been suggested as potential auto-antigens for MS. Although no specific causative protein has been established, T cells specific for MBP, proteolipid protein, MOG and myelin-associated glycoprotein have been found in MS patients (Sun et al., 1991a; Sun et al., 1991b; Meinl et al., 1993). Moreover, the finding of IL-12 and IFN-γ in CNS has revealed a critical role of Th1 cells in the pathogenesis of MS (Comabella et al., 1998). Similarly, B cells and antibodies specific to all kinds of myelin proteins such as MBP, proteolipid protein and MOG have been found in MS patients (Genain et al., 1999; Cross et al., 2001). The proposed importance of antibodies in the disease also suggests that the complement system has a role in MS. In accordance with this, active complement components have been found in lesions of MS patients (Gay and Esiri, 1991; Cross et al., 2001).

To date, there is no known treatment for MS and therapies mainly focus on immune suppression. MS has a complex inheritance pattern, and seems to be largely controlled by genetic factors (Ebers et al., 1996; Compston, 2000).

5.1. Experimental autoimmune encephalomyelitis (EAE)

EAE is the most commonly used animal model for MS in mice. Several antigens, such as whole spinal cord homogenates, MBP, proteolipid protein, MOG or even just peptides of the myelin proteins, emulsified in CFA can be used for induction of the disease (Pettinelli et al., 1982; Amor et al., 1994). MOG-induced EAE in rats has been suggested to resemble the complexities in immune effector mechanisms observed in MS (Weissert et al., 1998). In most EAE models, pertussis toxin is required (Munoz et al., 1984). It has been suggested that pertussis toxin opens up the blood-brain barrier (BBB), leading to an increased permeability (Linthicum and Frelinger, 1982).

The classical EAE symptoms are weakness in the tail, disturbance of hind limb mobility, paraplegia and disturbances of bladder and bowel function. Like MS, the disease course generally progresses along three paths: acute, chronic progressive or chronic relapsing (Lublin and Reingold, 1996). Histological studies of brain and spinal cord implicate plaques of tissue injury, where lymphocytes, plasma cells and macrophages are found. Axonal damage has not been observed, but the protective myelin sheets that surround the nerves have been reported to be destroyed (Martin et al., 1992; Cross et al., 2001). Activated monocytes/macrophages, which mainly perform the tissue destruction, are probably stimulated by IFN-γ secreted from myelin-specific Th1 cells (Bauer et al., 1994).
In contrast to the CIA model, transfer of CD4+ T cells can induce EAE and Th1 cells were found to be critical for the disease induction (Ben Nun et al., 1981; Ando et al., 1989). Interestingly, CD4 or CD8-deficient mice are susceptible to EAE (Koh et al., 1992; Koh et al., 1994). The role of B cells in the pathogenesis of EAE is not as clear as it is in the case of RA. For instance, B cell-deficient mice are susceptible to EAE after being immunized with peptides of MBP or MOG (Wolf et al., 1996; Hjelmstrom et al., 1998). Moreover, dual roles have been suggested for antibodies: on the one hand, antibodies are involved in the re-myelination process (Rodriguez and Lennon, 1990); and on the other, antibodies could activate the complement and help in opsonization to phagocytic cells in the demyelination process (Trotter et al., 1986). The exact role of the complement system is also unclear in EAE. C5-deficient mice are still susceptible to EAE (Nataf et al., 2000; Calida et al., 2001), whereas mice with the same deficiency are completely resistant to CIA (Wang et al., 2000; Ji et al., 2002).

5.2. Role of the plasminogen activator system during brain damage

It is well established that tPA and plasminogen have important roles in certain CNS pathologies related to brain damage (Gingrich and Traynelis, 2000). tPA and plasminogen are involved in neuronal plasticity, reorganization CNS tissues, and neuronal death (Tsirka et al., 1997a). They also mediate a critical step in the progression of excitotoxin-induced neurodegeneration (Seeds et al., 1995; Wu et al., 2000).

Mechanistically, tPA appears to function through proteolytic and non-proteolytic pathways. The proteolytic pathway proceeds via its well-established ability to convert plasminogen into plasmin (Werb, 1997; Chen et al., 1999). Plasmin promotes neuronal death via degradation of the ECM and the establishment of chemotactant gradients for microglia (Werb, 1997). For instance, plasmin was shown to degrade laminin soon after excitotoxic injury (Chen et al., 1999). Plasmin also facilitates neurite outgrowth through processing of the ECM proteoglycans. In the non-proteolytic pathway, tPA functions as an agonist to stimulate a cell-surface receptor on microglia, resulting in microglial activation. Once activated after neuronal injury, microglia contribute to the ensuing neurodegeneration (Rogove et al., 1999). Recently, it has been shown that tPA potentiates signaling mediated by glutamatergic receptors by proteolytically regulating N-methyl-D-aspartate receptors and thus increasing the influx of calcium (Nicole et al., 2001).

In 1981, it was demonstrated that tPA is released at the neuronal growth cone (Krystosek and Seeds, 1981). Studies on rodents indicate that tPA and plasminogen are synthesized locally in the brain. In addition, these molecules can enter brain tissue through a compromised BBB (Gingrich and Traynelis, 2000). However, it is the CNS-expressed tPA and plasminogen that promote neurodegeneration, rather than the influx of plasminogen via disrupted BBB (Chen et al., 1999). A morphological differentiation of neuroblastoma cells is accompanied by induction of tPA, suggesting that tPA also plays a role in neuronal cell functions (Neuman et al., 1989). A widespread expression of tPA has been found in the human CNS, in particular in neocortical mantle, thalamus, amygdala and hippocampal pyramidal neurons. In the hippocampus, it has been shown that plasminogen is synthesized by neuronal cells, whereas tPA is synthesized by both neurons and microglial cells (Tsirka et al., 1997b).

In addition to its role in neurodegeneration, tPA has been found to mediate microglial activation through some mechanism which is distinct from the conversion of plasminogen into plasmin.
Microglia are the immunocompetent cells of the CNS, and they function in both neuroprotection and neurotoxicity in the brain. They reside in a resting form (Giulian and Baker, 1986) and become activated following stimulation. The activation includes various changes to these cells such as migration to the site of injury, local proliferation, changes in gene expression and phagocytosis (Kreutzberg, 1996). Activated microglia are neurotoxic and inhibiting or delaying microglial activation can result in neuroprotection (Thanos et al., 1993; Rogove and Tsirka, 1998). Activated microglia are associated with several neuropathological conditions, such as Alzheimer’s disease (Mrak et al., 1995), stroke (Wood, 1995) and MS (Williams et al., 1994b). Accordingly, tPA-deficient microglia display attenuated activation in response to excitotoxic stimuli in vivo. In contrast, the microglial response to excitotoxic stimuli in plasminogen-deficient mice was comparable to that of wild-type controls (Tsirka et al., 1997a). In vitro cultured microglia deficient in tPA also display an attenuated activation in response to lipopolysaccharide (Rogove et al., 1999).

It is evident that tPA plays a critical role in the mammalian CNS. A physiological and tight control of tPA is therefore vital to normal CNS function and survival (Tsirka, 2002; Teesalu et al., 2002). During pathological events, signaling cascades both within neurons and among neurons, microglia and macroglia are initiated, which ultimately results in neuronal death. In this respect, understanding the interactions between these CNS cells, the different forms of neuronal injury, and also the steps that control tPA activity and release, will be critical in deciphering the role of tPA in the CNS.

5.3. **Role of the plasminogen activator system during multiple sclerosis**

Extracellular proteolysis in inflammatory demyelination is increasingly recognized as a pathogenic factor in demyelinating diseases such as human MS and its animal model, EAE (Cuzner et al., 1996; Cuzner and Opdenakker, 1999). Increased levels of MMPs have been found in the CSF of MS patients and EAE mice, and these enzymes are up-regulated in a pattern that indicates an active role in inflammation and demyelination (Lukes et al., 1999). In addition, plasmin inhibitors have been shown to suppress the development of EAE (Smith and Amaducci, 1982). It has also been shown that patients with MS have 10-times higher tPA activity in the CSF than the control group (Akenami et al., 1996; Akenami et al., 1997). High levels of complex between PAI-1 and uPA and plasmin-α2-AP complex, together with reduced plasminogen concentrations, have also been found in MS patients (Akenami et al., 1997; Akenami et al., 1999). In addition, neurons and glial cells synthesize neuroserpin which inhibits tPA (Hastings et al., 1997). Together, these results suggest that tPA and plasminogen activation are implicated in tissue-destructive processes in CNS.

Several possible mechanisms through which tPA may be acting in MS can be proposed. Firstly, plasmin can directly degrade MBP, suggesting that tPA may promote demyelination (Cammer et al., 1978). In addition, plasmin is a potent activator of the MMP cascade and MMP activity has been shown to have an important role in the breakdown of myelin membranes (Cuzner and Opdenakker, 1999). Secondly, tPA may alter inflammatory reactions in the CNS by increasing the permeability of the BBB (Paterson et al., 1987). Thirdly, because tPA can promote excitotoxic cell death, tPA may also play a role in the early stages of MS by contributing to glutamate-induced oligodendrocyte injury and neuronal death (Pitt et al., 2000). Lastly, tPA may help neuronal regeneration by reducing local fibrin deposition (Herbert et al., 1996; Akassoglou
et al., 2000) or by promoting migration of oligodendrocyte progenitors through the ECM (Uhm et al., 1998). Taken together, these observations suggest that the involvement of the PA system seems to be complex, as it may play both harmful and protective roles in the development of MS.

6. **Wound healing**

Skin is composed of three main layers: an outer keratinizing stratified squamous epithelium called the epidermis, an underlying strong supporting and nourishing layer of fibroelastic tissue called the dermis, and a variable deep layer of mainly adipose tissue called the hypodermis or subcutis (Richards et al., 2003). The major function of skin is to be the first protective barrier against the environment. Loss of the integrity of large portions of the skin as a result of injury or illness may lead to major disability, or even death. The primary goal of the treatment of wound is rapid wound closure, resulting in a functional and aesthetically satisfactory scar. Defect in wound healing is a large medical problem. For example, in the United States more than 1.25 million people have burns and 6.5 million have chronic skin ulcers caused by pressure, venous stasis, or diabetes mellitus (Singer and Clark, 1999).

Wound healing is a dynamic, interactive process involving soluble mediators, formed blood elements, ECM, and parenchymal cells. The repair processes involved in wound healing follow a specific time sequence, and can be temporally categorized into three major groups: inflammation, tissue formation, and tissue remodeling (Singer and Clark, 1999). However, these events overlap somewhat in time.

6.1. **Inflammation during wound healing**

Severe tissue injury results in disruption of blood vessels, extravasation of blood constituents, blood coagulation and formation of a provisional ECM rich in fibrin which facilitates cell migration. In this process, platelets have a dual function, since they not only facilitate the formation of a hemostatic plug but also secrete several mediators such as platelet-derived growth factor (PDGF), which stimulate the healing process (Heldin and Westermark, 1999). In the absence of hemorrhage, platelets are not essential for wound healing. The coagulation and complement pathways, and also activated parenchymal cells, all generate numerous vasoactive mediators and chemotactic factors, which together recruit inflammatory leukocytes to the wound area (Singer and Clark, 1999).

Within 24 hours of the incision, infiltrating neutrophils appear at the margins of the incision. The neutrophils move toward the fibrin clot, cleanse the wounded area of foreign particles and bacteria, and are then extruded with the eschar or phagocytosed by macrophages or fibroblasts (Diegelmann and Evans, 2004). In response to specific chemoattractants such as TGF-β and specific fragments of ECM protein, circulating monocytes also infiltrate the site of wound and become activated macrophages that release growth factors such as PDGF and vascular endothelial growth factor to initiate the formation of granulation tissue. Macrophages bind to specific proteins of the ECM by their integrin receptors to stimulate phagocytosis of microorganisms (Brown, 1995). These activated macrophages express TNF-α, TGF-α, TGF-β, and colony-stimulating factor 1, a cytokine necessary for the survival of macrophages (Rappolee et al., 1988; Shaw et al., 1990). The monocyte-derived growth factors are almost certainly necessary for the initiation and propagation of new tissue formation in wounds (Leibovich and Ross, 1975),
wereas macrophages, especially, appear to have a pivotal role in the transition between inflammation and repair (Singer and Clark, 1999).

6.2. **Re-epithelialization during wound healing**

Re-epithelialization of wounds begins within hours after an injury. Epithelial cells from residual epithelial structures quickly remove the clotted blood and damaged stroma from the wound space. In the skin, the keratinocytes of the stratified epidermal sheet appear to move over each other in a leapfrog fashion (Winter, 1962). Concomitant with migration, keratinocytes undergo a marked metamorphosis that includes retraction of intracellular tonofilaments, dissolution of most intercellular desmosomes and formation of peripheral cytoplasmic actin filaments (Gabbiani et al., 1978; Goliger and Paul, 1995). The dissolution of hemidesmosome links between the epidermis and the basement membrane results in detachment of epidermal cells and their lateral movement. The expression of integrin receptors on epidermal cells allows them to interact with a variety of ECM proteins (e.g. fibronectin and vitronectin) in the margin of the wound and the fibrin clot in the wound space (Clark, 1990; Larjava et al., 1993). The degradation of the ECM is dependent on collagenases produced by epidermal cells, and also plasmin which activates several MMPs (Bugge et al., 1996; Creemers et al., 2000).

One to two days after an injury, epidermal cells that are present at the wound margin – but behind the actively migrating cells – begin to proliferate. However, cell migration does not depend on cell proliferation (Bertone, 1989). Several mechanisms exist for the stimulation of epidermal cell proliferation during re-epithelialization including chemotactic factors, active contact guidance, loss of nearest-neighbor cells, or a combination of these processes (Brown et al., 1986; Barrandon and Green, 1987). Interestingly, TGF-β can promote the outgrowth of epidermal cells from organ cultures despite the fact that it is a potent inhibitor of keratinocyte proliferation *in vitro* (Hebda, 1988).

As re-epithelialization continues, basement membrane proteins re-appear in a very ordered sequence from the margin of the wound inwards, in a zipper-like fashion (Clark et al., 1982). Epidermal cells revert to their normal phenotype, firmly attaching to the re-established basement membrane through hemidesmosomes, and underlying dermis through type VII collagen fibrils (Gipson et al., 1988).

6.3. **Remodeling of granulation tissue during wound healing**

Approximately four days after injury, new granulation tissue begins to invade the wound space. Macrophages, fibroblasts and blood vessels move into the wound area simultaneously as one unit. Macrophages provide a continuous source of growth factors necessary to stimulate fibroplasia and angiogenesis (Singer and Clark, 1999).

Growth factors, especially PDGF and TGF-β1, together with molecules from the ECM, stimulate fibroblasts of the tissue around the wound to proliferate and express the appropriate integrin receptors that are required for the migration into the wound area (Xu and Clark, 1996; Branton and Kopp, 1999). This process likely requires an active proteolysis that can cleave a path for cell migration. The PA and MMP systems are potential candidates for this task (Lund et al., 1999). Fibroblasts are responsible for the synthesis, deposition and remodeling of the ECM. Fibroblasts
and the newly-formed ECM from the granulation tissue are also called fibroplasia. The provisional ECM is gradually replaced with the collagenous fibroplasia, perhaps as a result of the action of TGF-β1 (Clark et al., 1995). Once an abundant collagen matrix has been deposited in the wound, the fibroblasts stop producing collagen. Cells in the wound undergo apoptosis, by which fibroblast-rich fibroplasia is replaced by a relatively acellular scar (Desmouliere et al., 1995).

Formation of new blood vessels (angiogenesis), is necessary to provide the newly formed granulation tissue with oxygen and nutrients for cell metabolism. The induction of angiogenesis is attributed to many molecules, including vascular endothelial growth factor, TGF-β and basic FGF (bFGF) (Folkman and D'Amore, 1996; Iruela-Arispe and Dvorak, 1997). bFGF may set the stage for angiogenesis during the first three days of wound repair, whereas vascular endothelial growth factor is critical for angiogenesis during the formation of granulation tissue from day 4 to day 7 (Nissen et al., 1998). It has been suggested that protease activity is also required for angiogenesis (Pintucci et al., 1996). Once the new granulation tissue has formed, angiogenesis ceases and many of the new blood vessels disintegrate by apoptosis (Ilan et al., 1998). Angiostatin has been suggested to be involved in regulating the apoptosis process (Folkman, 1997), although more studies are required to confirm this finding.

6.4. Wound contraction and scar tissue formation

Contraction of wounds involves a complex and finely-balanced interaction between cells, ECM, and cytokines. During the second week of healing, the wound is filled with granulation tissue and covered with a new epidermis. By then, fibroblasts have been transformed into myofibroblasts, characterized by large bundles of actin-containing microfilaments located along the cytoplasmic face of the plasma membrane (Risau, 1997). These myofibroblasts contract the wound and epidermal cells differentiate to re-establish the permeability barrier. The contraction involves attachment of fibroblasts to the collagen matrix through integrin receptors (Schiro et al., 1991) and formation of cross-links between individual bundles of collagen (Woodley et al., 1991). The collagen remodeling during the transition from granulation tissue to scar tissue is dependent on continued synthesis and catabolism of collagen at a low rate. This process is controlled by several MMPs secreted by macrophages, epidermal cells, and endothelial cells, and also fibroblasts (Mignatti and Rifkin, 1996b). Nevertheless, a healed wound can never attain the same breaking strength as uninjured skin. At maximal strength, a scar is only 70% as strong as normal skin.

6.5. Role of the plasminogen activator system during wound healing

During healing of wounds, proteolytic activity is required in many processes including inflammation, provisional matrix removal, formation of granulation tissue, matrix formation, and also for migration of keratinocyte from the wound edges towards the centre of the wound. The involvement of the PA system in wound healing has been well established (Vassalli and Saurat, 1996; Li et al., 2003). PAs are produced by keratinocytes, fibroblasts, capillary endothelial cells, inflammatory granulocytes and macrophages (Romer et al., 1991). The PAs exert their roles by dissolving the fibrin clot, degrading ECM, mobilizing and activating growth factors, facilitating cell migration, and promoting angiogenesis.
The importance of plasmin was demonstrated in a skin wound healing study in which plasminogen-deficient mice were challenged with incision wounds (Romer et al., 1996). It was found that plasminogen-deficient mice had a striking delay in wound healing, with impaired keratinocyte migration. However, inflammatory cell migration and granulation tissue remodeling were not compromised. It was concluded that plasminogen function was eventually taken over by other factors, resulting in a delay in the healing. When a similar study was performed on plasminogen/fibrinogen doubly deficient mice, the healing time was corrected (Bugge et al., 1996). Therefore, these authors suggested that the fundamental and possibly only essential role of plasminogen is to degrade the fibrin-rich provisional matrix. In another study where wild-type mice were treated with the MMP inhibitor galardin, a similar retarded wound healing as seen in plasminogen-deficient mice was found. When wild-type mice were treated with galardin the wounds were healed within 60 days. When plasminogen-deficient mice were treated with galardin, however, healing was completely arrested and wound closure was not seen during an observation period of 100 days (Lund et al., 1999). Based on these data, the authors proposed that there is a functional overlap between the PA and MMP systems, probably in the dissection of the fibrin-rich provisional matrix by migrating keratinocytes (Lund et al., 1999).

In our study of the healing of TM perforations (Paper IV), we found that the healing was permanently impaired in plasminogen-deficient mice. Several of the dynamic interactive processes associated with a successful TM healing were disturbed in the plasminogen-deficient mice. These results contrast with the study on skin wound healing. In that study, the wound healing in plasminogen-deficient mice was impaired but eventually proceeded, leading to delayed but complete healing in all mice (Romer et al., 1996; Lund et al., 1999). In fact, when reconstitution of plasminogen in plasminogen-deficient mice was performed on a permanent perforation wound, normal healing of the TMs could be accomplished within 7 days (unpublished data). In our study of the long-term healing pattern of skin incision wounds, plasminogen-deficient mice demonstrated a permanently abnormal healing pattern, with a persistent deposition of necrotic tissue beneath the closed epidermis (unpublished manuscript). These data suggest that the essential role of plasminogen during wound healing is still debatable and different healing patterns may exist in different organs of the body.

7. Wound healing in the middle ear

The middle ear in rodents resembles the human middle ear. However, in contrast to humans, rodents lack a mastoid system with air-filled cells. Instead, they have a large middle-ear bulla. Over the past 20 years, the rat has become the most common rodent model for the study of otitis media (OM) (Alper et al., 2002). The discussion following in the rest of this chapter is based mainly on rat anatomy.

The middle ear is an air-filled cavity, the tympanic cavity, located in the petrous temporal bone that is separated from the external auditory canal by the TM. Sound waves impinging on the TM are converted into mechanical vibrations which are then amplified by a system of levers made of the three small bones, ossicles (the malleus, incus and stapes), and transmitted to the fluid-filled inner ear cavity (Belin et al., 1998). The ossicles articulate with one another via synovial joints and the malleus and incus pivot on tiny ligaments which are attached to the wall of the middle-ear cavity. Anteriorly, the middle-ear cavity communicates with the nasopharynx via the auditory (Eustachian) tube, which permits equalization of pressure changes with the external environment.
(Albiin et al., 1983). Most of the middle ear and mastoid cavities are lined with a simple respiratory squamous epithelium (Lim and Hussl, 1969). Two tracts with a cylindrical ciliated and goblet cells emanate from the tympanic opening of the Eustachian tube and extend superior and inferior to the promontory (Albiin et al., 1986).

The mucosal lining of the middle ear is continuous with the respiratory mucosa of the nasopharynx (Sade, 1966). The sub-epithelial loose connective tissue with its blood vessels, nerves, and various connective tissue cells has been suggested to be involved in the pathogenesis of certain middle-ear disease, such as OM (Ruah et al., 1995; Alper et al., 2002). In the case of OM with effusion, it has been supposed that an increased production of fluid/mucus, exudate from blood vessels, and an increased secretory activity of the mucosa occur (Tos, 1980). In the process of clearance of fluid from the middle ear, the effusion material can be removed by transport through the Eustachian tube, or by absorption through the mucosa (Lim, 1974).

7.1. The tympanic membrane (TM)

The TM receives, augments and transmits sound waves to the hearing ossicles, and also functions as a physical barrier to protect the middle ear from injury. The TM consists of three distinct layers, an outer keratinized stratified squamous epithelium, a connective tissue layer, and an inner flat and single layered epithelium which is contiguous with the epithelial lining of the middle-ear cavity (Lim, 1968a; Lim, 1968b; Lim, 1970). Anatomically, the TM is divided into the pars tensa (PT) and the pars flaccida (PF), which differ regarding both histology and function.

The PT is the acoustic portion of the TM. In the rat, the thickness of PT is 5-10 µm (Schmidt and Hellstrom, 1991). The collagen fibers in the collagen layer form two layers: an outer layer of densely packed bundles of collagen fibers running radially from the handle of the malleus to annulus fibrosus, and a looser inner layer of annularly oriented fibers (Lim, 1968a; Schmidt and Hellstrom, 1991). There is also a thin layer of connective tissue beneath the epidermal and mucosal epithelium (Lim, 1968a).

The thickness of the rat PF is approximately 50 µm (Widemar et al., 1984). The connective tissue layer differs from that of the PT in that it is an irregularly-arranged and loose connective tissue (Lim, 1968b; Lim, 1970). The PF is extremely rich in mast cells which are localized subepidermally close to nerves and vessels (Albiin et al., 1985). The physiological role of the PF is still not fully understood. A role in pressure equalization of the middle-ear cavity has been suggested (Hellstrom and Stenfors, 1983). Experimental studies have shown that the PF responds to inflammatory stimuli earlier than the PT, irrespective of the causative agent (Goldie and Hellstrom, 1986; Magnuson and Hellstrom, 1994).

The vessels supplying the TM derive from the external carotid artery (Judkins and Li, 1997). They form a fine network around the annulus fibrosus, and some larger vessels are situated along the lining of, and also spreading into, the PF and the handle of the malleus (Judkins and Li, 1997). The centre of the PT, being devoid of vessels, is dependent on passive diffusion (Hellstrom et al., 2003). After mechanical stimulation or pathological conditions, the TM vessels dilate, and at least part of the dilation may be due to release of vasoactive substances from abundant nerves and/or mast cells located close to the vessels (Hellstrom et al., 2003).
The term “myringotomy” is formed from two words: myringa, the Latin word for the TM; and tomy, meaning cutting. Surgically, myringotomy is used for creating an incision of the PT to remove fluid (often infected) from the middle-ear cavity, in cases of chronic or recurrent OM. When myringotomy is performed in the rat, an intense and early inflammatory reaction is initiated in the TM. The inflammatory reaction occurs first in the PF and later in the PT (Mattsson et al., 1999). After myringotomy the rat TM usually heals within 9-11 days. During healing of a perforation in the PT, a keratin spur advances ahead of an in-growing and remodeling epithelium and connective tissue (Stenfors et al., 1980; Stenfors, 1987).

7.2. Tympanic membrane perforations as a model of wound healing

Clinically, TM perforations are a frequent cause of conductive hearing loss. Symptoms of TM perforations include mild conductive hearing loss, aural fullness and mild tinnitus. TM perforations can be acute or chronic. An acute perforation may happen secondary to an episode of acute OM, or in relation to a trauma (Teel et al., 1989; Bluestone, 2000). The cause of a traumatic injury to the TM is usually a sudden increase in air pressure in the external ear canal, or direct penetration of the TM by a foreign object. The majority of these perforations heal spontaneously, involving proliferation of keratinizing squamous epithelium advancing ahead of an in-growing connective tissue (Mattsson et al., 1998; Mattsson et al., 1999). However, the reason why some perforations heal, whereas others stay open, is still an open question.

Experimental perforations of the TM have been proposed to be used as a wound healing model (Hellstrom et al., 1991). However, there are differences between skin wound healing in general and healing of the PT after myringotomy. The transparent portion of the PT is normally devoid of vessels, indicating that there is a decreased leakage of blood components to the site of injury. In skin wound healing, the tissue injury immediately causes a disruption of blood vessels and extravasation of blood constituents. The blood clot provides a provisional extracellular matrix for cell migration. However, due to anatomical differences, there is no underlying stromal tissue in the TM. Therefore, there is no apparent formation of blood clot and provisional matrix at the perforation. Instead, the keratinized squamous epithelium forms a guiding keratin spur and advances ahead of an in-growing and remodeling connective tissue. Furthermore, reformation of the fibrous component of the TM occurs after that the migratory epithelium has sealed the perforation (Reijnen and Kuijpers, 1971). The healing of TM perforations is a well-organized chain of inflammatory events, with an initial invasion of inflammatory cells followed by reparative and restoration phases (Mattsson et al., 1997). Although variations exist concerning the exact speed of healing, a general healing pattern is described below (Mondain and Ryan, 1993; Fina et al., 1993).

The healing process starts from the PF within hours of the injury. The PF responds immediately with an inflammatory reaction characterized by neutrophils scattered in the connective tissue layer. The neutrophils predominate at around 6 hours after the perforation. Thereafter, an abundant influx of macrophages can be observed. The accumulation of macrophages culminates about 12 to 24 hours after the perforation. The inflammatory response of the PT occurs in the vascularized connective tissue layer between the handle of the malleus and the outer epithelium (Mattsson et al., 1999). Vessels which are normally seldom seen in the semi-transparent portion of the TM will then appear at the border of the perforation. These vessels, most likely represent dilated pre-existing vessels. Also, the edge of the perforation is formed by the fibrous layer either
exposed to or surrounded by infiltrating cells or a fibrin exudate. Epithelia and fibroblasts begin to react near the sulcus or the malleus. The connective tissue is not well organized initially and grows under the epithelial layer.

At day 3 or 4, the fibrous layer is covered by infiltrating neutrophils and by the keratin layer. Close to the edge of the perforation, there is prominent proliferation of the epithelial layer. Proliferation of the fibroblasts is seen in the sub-epithelial layer. This process is accompanied by the progression of a material made up of fibrin, keratin and infiltrating cells forward to or through the perforation. An in-growth of newly formed blood vessels begins (Hellstrom et al., 2003). At day 7, the perforation is near to being closed by epithelial cells and a keratin layer. The connective layer contains young fibroblasts, as well as vessels and infiltrating cells. Fibroblast hyperplasia is located primarily at the edge of the perforation. At this time, the amount of ECM is still limited. At day 10, the healed TM is still thicker than the normal membrane and the connective layer is hypertrophic, without evidence of any fibers. Blood vessels are numerous in the connective tissue present in the healed part of the membrane. Mucosal cells are visible, and keratin is present close to the edges of the fibrous layer.

The powerful ability of the TM to heal mainly rests upon the migratory capacity of the outer keratinizing squamous epithelium. Migration of the TM squamous epithelium is a well-recognized phenomenon, characterized by a marked keratin production. The keratin forms a spur, acting as a guide, ahead of the advancing epithelium. Once the keratinizing squamous epithelium is closed, it is thinned out and then the rims of the fibrous layer reach each other – with a final closing of the mucosal epithelium. Normally a residual scar will disappear within a few weeks.

7.3. Methods for improvement of TM wound healing by topical treatment

With a view to accelerating the closure of TM perforations, research has targeted two different mechanisms. One is to provide additional physical support for guiding regenerating tissue. Paper patching has been used, but with a low success rate (Laurent et al., 1991). In experiments on rats, hyaluronan has also been used to increase the healing rate and improve the quality of the healed TM perforations (Laurent et al., 1988). Other biological materials, including the patient’s own blood as a fibrin adhesive (Siedentop et al., 1986), Gelfilm and biodegradable materials (Baldwin and Loftin, 1992), and lysates of cultured keratinocytes (Somers et al., 1996) have also been used with some success – both in animal models and in limited clinical studies.

The other method to accelerate the closure of TM perforations is to induce cellular replication and migration. Since growth factors control the proliferation and migration of cells that modulate re-epithelialization, angiogenesis and collagen metabolism, extensive animal studies have been performed to determine the effects of different growth factors in TM perforations. EGF, basic FGF, PDGF and TGF-α have been shown to be induced by acute TM perforation (Koba and Kawabata, 1995; Mondain and Ryan, 1995; Yeo et al., 2000). Expression of receptors for EGF, basic FGF, PDGF and keratinocyte growth factor in the TM has also been reported (Mondain and Ryan, 1995; Lim, 1995; Ma et al., 2002). However, further experimental and clinical studies on the mechanism of action of growth factors, timing of application, selection (either singly or in combination), mode of delivery, dose and safety aspects – as well as more clinical trials – will pave the way for clinical application of such growth factors (Ma et al., 2002).
8. Infection

Infectious diseases are caused by pathogens such as bacteria and viruses. Pathogenic bacteria may contain virulence factors that mediate interactions with the host, eliciting particular responses from the host cells that promote the replication and spread of the pathogen. Viruses rely entirely on subverting the machinery of the host cell to produce their proteins and to replicate their genomes. Pathogens often colonize the host by adhering to or invading the epithelial surfaces that are in direct contact with the environment. Viruses rely largely on receptor-mediated endocytosis for host cell entry, while bacteria exploit cell adhesion and phagocytic pathways (Aderem, 2003).

During the first critical hours and days of host exposure to a new pathogen, the innate immune system is the first line of defense against invading pathogens. However, the initiation of specific adaptive immune responses is also required (Davenport et al., 2003). Innate immune responses rely on the body’s ability to recognize conserved features of pathogens that are not present in the uninfected host. These include many types of molecules on microbial surfaces and the double-stranded RNA of some viruses (Levy et al., 2003; Basset et al., 2003). Surface molecules of microorganisms also activate the complement system to target these organisms for phagocytosis by macrophages and neutrophils, and to produce an inflammatory response (Lachmann and Davies, 1997).

Bacteria have developed different strategies to escape from phagocytes. For instance, they can inhibit chemotaxis and phagocytosis, kill or colonize the phagocytes (Chensue, 2001; Supuran et al., 2002). The phagocytic cells use a combination of degrading enzymes, anti-microbial peptides and reactive oxygen species to kill the invading microorganisms (Hauschildt and Kleine, 1995). In addition, they release signaling molecules that trigger an inflammatory response and begin to marshal the forces of the adaptive immune system (Chertov et al., 2000). Bacteria, on the other hand, have developed different strategies directed against the adaptive immune system such as molecular mimicry, suppression of antibodies, hiding inside cells, or release of antigen into the bloodstream (Chertov et al., 2000).

8.1. Role of the plasminogen activator system during bacterial infection

The PA system has been suggested to be involved at several stages and by various mechanisms during both bacterial invasion and host defense (Lahteenmaki et al., 2001). A vast number of pathogens express plasmin(ogen) receptors (Broder et al., 1991; Berge and Sjobring, 1993). Bacteria also influence the secretion of PAs and their inhibitors from mammalian cells (Brandtzaeg et al., 1990; Fuchs et al., 1996). For instance, production of uPA has been found to be enhanced in cells infected by various bacteria (Fuchs et al., 1996). The bacterial PAs streptokinase and staphylokinase are not enzymes themselves, but they form 1:1 complexes with plasminogen and plasmin, and thus acquire a remarkable efficiency to activate plasminogen (Parry et al., 2000). To date, in vivo evidence for a role of plasminogen activation in pathogenesis exists in a few bacteria such as Yersinia pestis, Borrelia, and group A streptococci (Sodeinde et al., 1992; Li et al., 1999; Gebbia et al., 1999; Goguen et al., 2000).

The Pla surface protease of Yersinia pestis functions like the mammalian PAs, and activates plasminogen by limited proteolysis at the same Arg\textsuperscript{560}-Val\textsuperscript{561} bond as tPA and uPA do (Lahteenmaki et al., 2001). Binding of plasminogen to receptors present on the surfaces of some
bacteria convert these bacteria into proteolytic organisms. In Gram-negative bacteria, the filamentous surface appendages form a major group of plasminogen receptors (Lahteenmaki et al., 1993; Klemm and Schembri, 2000). In Gram-positive bacteria, surface-bound molecules have been identified as plasminogen receptors (Berge and Sjobring, 1993; Pancholi and Fischetti, 1998). As a consequence, plasmin can be generated on the surface of microorganisms such as *Haemophilus influenzae*, *Salmonella typhimurium*, *Streptococcus pneumoniae*, *Yersinia pestis*, and *Borrelia burgdorferi*, which can lead to a degradation of mammalian ECM (Sodeinde and Goguen, 1989; Lahteenmaki et al., 1995; Coleman et al., 1995; Virkola et al., 1996). Furthermore, bacterial proteases may also directly activate latent pro-collagenases or inactivate protease inhibitors in human plasma, and thus contribute to tissue damage and bacterial spread across tissue barriers (Harrington, 1996; Paul et al., 1998).

8.2. Infections of the middle ear

Inflammation of the middle ear, or otitis media (OM), is one of the most common diseases in childhood. OM is not a single disease entity; it includes two main inflammatory conditions, acute OM, and OM with effusion (Paparella, 1976). Acute OM is defined as an inflammation of the middle ear with a purulent effusion and with concomitant symptoms such as fever, otalgia or irritability. Clinical findings in an acute OM are a reddish, bulging TM and a pus-filled middle-ear cavity (Bluestone and Klein, 1984). The incidence of acute OM shows seasonal variations, culminating during the cold months. The three most frequent bacterial causes of acute OM are *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* and they are found in 35-51%, 12-23% and 6-14% of the acute OM cases, respectively (Kamme et al., 1971; Bluestone, 1992). The pathogenic bacteria ascend through the Eustachian tube to cause acute OM, and the adenoid has been suggested to be the primary site of these bacteria (Gates et al., 1992; Bernstein et al., 1994). A low-grade virus infection may cause a primary dysfunction of the Eustachian tube (Gates et al., 1992; Bernstein et al., 1994).

OM with effusion is an inflammation of the middle ear with liquid collected in the middle-ear space. The signs and symptoms of an acute infection are absent, and there is no perforation of the TM (Bluestone and Klein, 1984). A prerequisite for OM with effusion to develop is an inflammation of the middle-ear mucosa. For many years, the main hypothesis for the pathogenesis of OM with effusion was Eustachian tube dysfunction. More recent investigations have indicated that the major cause is infection. In the middle-ear fluid, bacteria, lymphocytes, lysosomal enzymes, histamine, immunoglobulin and various cytokines have been found (Juhn and Jung, 1985; Yellon et al., 1995). When isolates of material from middle-ear effusion were cultured, 16-30% of them have shown to have positive growth of bacteria. The most frequent bacteria are *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* (Kamme and Nilsson, 1984; Mills et al., 1985; Stenfors and Raisanen, 1992). However, more recent studies using PCR have shown that at least fragments of these pathogens can be found in almost all the middle-ear fluids in OM with effusion.

8.3. Role of the plasminogen activator system during ear infection

Human and bacterial proteases have long been known to play a role in the pathogenesis of OM (Juhn et al., 1976). Leukocyte-derived proteases can help to prevent or eradicate bacterial infections, but they may also contribute to tissue damage in OM, causing sequelae or disease
persistence (Granstrom et al., 1987; Avidano et al., 1998). As discussed earlier, the PA system plays an important role in bacterial invasion over tissue barriers. On the other hand, the PA system is also important for inflammatory cell migration in order to clear bacteria, and for tissue remodeling during infection. It has also been suggested that the PA system plays a role during ear infection (Ohsaki et al., 1985; Salonen et al., 1989). In fact, PAs have been found in middle-ear effusions (Hamaguchi and Sakakura, 1992; Avidano et al., 1998). Studies on children with secretory OM indicate high levels of plasmin in mucoid effusion material, which suggest that there is an ongoing proteolytic process in secretory OM (Salonen et al., 1989). In our study of spontaneous episodes of OM (Paper V), virtually all the plasminogen-deficient mice in the study developed severe OM over an 18-week period, whereas basically all the wild-type control mice kept an uninfected middle-ear status. These findings suggest that plasminogen plays an essential role in host defense mechanisms against bacterial infection in OM.

A cholesteatoma is an epidermal structure. It grows independently, gradually replaces the middle-ear mucosa and resorbs underlying bone (Sculerati and Bluestone, 1989). It tends to recur after removal (Palva and Makinen, 1983). A cholesteatoma may develop secondary to secretory OM or chronic OM in a continuous process. Immunohistochemical studies have shown that plasminogen, uPA and PAI-1 are expressed in the cholesteatoma matrix, but not in a healthy TM. It has been suggested that an imbalance in the proteolytic activity of the cholesteatoma matrix may at least partly account for the aggressive behavior of this tumor-like lesion (Schonermark et al., 1999).

Access to animal models for middle-ear infections including OM, cholesteatoma and external otitis provides us with unique opportunities to study the participation of the PA system and the mechanisms that regulate matrix remodeling at the molecular level in middle-ear diseases. Such studies using animal models for these diseases in mice lacking different members of the PA system are under way in our laboratory.

9. The plasmin-complement functional correlation theory

Discrimination of self and non-self is one of the most fundamental issues for survival in all forms of living organisms. Primitive organisms have relied to a greater extent upon innate mechanisms of non-self recognition, whereas more evolved organisms have generally developed so-called adaptive immune responses (Zarkadis et al., 2001). The complement system is an innate mechanism of non-self recognition for defense against microbial agents. Complement activities have been detected in the most ancient group of vertebrates (the jawless fish), as well as in some invertebrate species (Sunyer et al., 1998). Several lines of evidence have suggested that the complement system has existed for at least 600–700 million years, which is 150–250 million years earlier than the emergence of adaptive immunity (Lambris et al., 1999). It seems that a primitive complement system appeared first, which formed a pathway that was the ancestor of the alternative and lectin pathways in vertebrates, while the classical pathway presumably emerged after the appearance of elasmobranchs and before the divergence of the teleost fish (Zarkadis et al., 2001).

Studies in invertebrates have provided a link between recognition of microbial molecular patterns, proteolytic cascades and activation of host defense, suggesting a common origin for the complement and coagulation cascades (Muller et al., 1999). Studies of molecular evolution has
shown that the complement and coagulation cascades are descendants of an ancestral defense system that served the dual role of immobilization and destruction of invading bacteria and the prevention of loss of body fluids (Patthy, 1990). The fibrinolytic and tissue remodeling protease cascades form another distinct group more closely related to the proteases of the digestive tract. Studies of the molecular evolution of these enzymes suggest that these enzymes are descendants of an ancestral protease responsible for degradation of ECM (Neurath, 1984; Patthy, 1990). It has been shown that the regulatory extensions of the proteases of the blood coagulation, fibrinolytic and complement cascades are derived from domains borrowed from other proteins (Patthy, 1990).

The complement system consists of a complex group of serum proteins, glycoproteins and soluble or membrane-bound receptors (Muller-Eberhard and Schreiber, 1980). Complement can be activated through three pathways: the classical, the alternative, or the lectin pathway. The three pathways converge at the point of activation of C3. Antigen-antibody complexes initiate the activation of the classical pathway, whereas the alternative and lectin pathways are activated in an antibody-independent fashion through interaction of complement components with specific carbohydrate groups and lipopolysaccharides present on the bacterial surface (Muller-Eberhard and Schreiber, 1980). Complement activation proceeds in a sequential fashion (Figure 2). This happens through the specific proteolytic cleavage of a series of proteins, and leads to the generation of active products that mediate various biological activities through their interaction with specific cellular receptors and other serum proteins. During the course of this cascade, the complement components initiate various biological processes including inflammation, leukocyte migration, and phagocytosis of complement-opsonized particles and cells (Mastellos et al., 2004). Currently, more than 30 complement proteins have been identified in plasma and on cell surfaces, and deficiencies in any particular component have been frequently associated with a diminished ability to clear circulating immune complexes or to fight infection (Walport, 2001).

The complement system plays a central role in innate immunity (Fearon and Locksley, 1996). It not only participates in inflammation and host defense, but also acts to link the innate immunity to the activation, regulation and the effector arms of the adaptive immunity (Fearon, 1998; Carroll, 2000). Although the traditional function of the complement system is to recognize and eliminate pathogens through direct killing (Moffitt and Frank, 1994) and phagocytosis (Brown, 1991), recent studies have shown that complement also plays a central role in enhancing humoral immunity of T cell-dependent and T cell-independent antigens (Carroll, 2000; Haas et al., 2002). Thus, the complement system can both modify cellular immunity and regulate tolerance to certain self antigens (Prodeus et al., 1998; Carroll, 2000; Kaya et al., 2001).

During the past few years, genetically modified mice have been generated by several groups with deficiencies in complement activation proteins, receptors and regulatory proteins. Many surprising findings have been obtained by using such mice. Accordingly, the complement system has been increasingly recognized to be involved in many tissue injury-repair processes. These processes include autoimmune diseases such as RA (Wang et al., 1995), MS (Hartung et al., 1992), systemic lupus erythematosus (Pickering et al., 2000), myocarditis (Kaya et al., 2001), and asthma (Humbles et al., 2000). Studies have also shown that the complement system may have novel roles in non-autoimmune disease processes such as bone and cartilage development (Maeda et al., 2001), mammalian reproduction (Xu et al., 2000), limb regeneration (Rio-Tsonis et al., 1998), liver regeneration (Mastellos et al., 2001), hematopoiesis and vascular development (Petrenko et al., 1999), and sepsis (Czermak et al., 1999).
The activation of complement components includes binding reactions and allosteric interactions, as well as enzymatic processes (Cooper, 1973). Certain complement proteins exhibit weak attractions for each other, and thus reactive sites are generated or uncovered in the component molecules. This interaction permits the activated components to interact with and activate the next component in the reaction sequence (Cooper, 1973). Enzymatic processes are important in the activation of most of the early-reacting components of the three pathways. Activation may be induced by intrinsic complement enzymes such as C3 and C5 convertases (Reid and Porter, 1981). Many of the complement components may also be activated in vitro by extrinsic or non-complement enzymes such as trypsin (C1, C4, C2, C3, C5), plasmin (C1, C3, C5) and bacterial enzymes (C3, C5) (Cooper, 1973).

Figure 2. Pathways of the complement system. Modified from Mollnes et al., 2002.

Whether the complement system can be proteolytically activated by plasmin in vivo is an interesting question that remains to be answered. A number of previous studies have shown that plasmin can cleave and/or activate several different components of the complement system in vitro in purified systems. Plasmin has been shown to cleave C1s to C1 esterase in vitro (Ratnoff and Naff, 1967). Plasmin can also cleave C3 after complement activation in vitro, although
plasminogen-depleted serum can still produce the cleavage (Lachmann et al., 1982). Active plasmin also has been shown to cleave C5 to generate C5a and C5b activity (Arroyave and Muller-Eberhard, 1973; Wetsel and Kolb, 1983). Among the terminal complement components C5b6, C7, C8 and C9, the non-enzymatic component C7 has been shown to be a plasminogen-binding protein. The consequence of plasminogen binding to C7 may therefore be two-fold, with PA-catalyzed plasminogen activation being increased and the bound plasmin being protected against inhibition by α2-antiplasmin (Reinartz et al., 1995). In another study, complement C5b-9 has been shown to increase the binding to and activation of plasminogen in human endothelial cells (Christiansen et al., 1997). A direct induction of complement activation by addition of plasminogen was observed in plasma and whole blood ex vivo (Schaiff and Eisenberg, 1997). Furthermore, plasmin can also inactivate complement inhibitors. For instance, C1 inhibitor is a pivotal inhibitor of protease activity in the early phase of the inflammatory response (Figure 2). It has been suggested that local degradation of C1 inhibitor by plasmin may be a central and critical event in the loss of protease inhibition during inflammation (Harpel, 1970; Wallace et al., 1997). A recent study showed that mast cells may participate in the modulation of the balance between proteases and protease inhibitors regulating tissue injury and repair (Wojta et al., 2002). Besides the effects of plasmin on complement activation, it also has been shown that plasmin causes inactivation of C2 and C4. Among the complement factors, C3 appears to be the most resistant to inactivation by plasmin (Pillemer et al., 1953). However, despite the fact that (based on the in vitro and ex vivo data) various possibilities of interaction between the PA system and the complement system have been proposed, the biological role of an interaction between the two systems in vivo has not been convincingly demonstrated.

In my studies, some of the obtained results showed that plasmin may play a critical role in the complement activation step in some autoimmune diseases (Papers I-III). Recently, while reading the literature, I noticed that many phenotypes of plasminogen-deficient mice are similar to the phenotypes of the complement-deficient mice during various non-autoimmune disease processes. For example, both plasminogen-deficient mice and C3 or C5-deficient mice showed similar liver pathomorphology after CCl4 injury (Bezerra et al., 1999; Mastellos et al., 2001; Strey et al., 2003; Markiewski et al., 2004). Traditionally, the phenotypes of the plasminogen-deficient mice during various diseases have mainly been attributed to an inability to proteolytically degrade fibrin or an impairment of tissue remodeling in these mice (Bugge et al., 1996; Ploplis, 2001). However, many results obtained by studies of plasminogen-deficient mice cannot be adequately explained by an inability to degrade fibrin. For instance, necrosis occurs extensively in different wound healing models in plasminogen-deficient mice (Bezerra et al., 1999; Creemers et al., 2000; Drew et al., 2000b), and this phenotype seems to be fibrin-independent (Bugge et al., 1996; Drew et al., 2000b). In addition, in our studies on the role of plasminogen during bacterial arthritis, plasminogen-deficient mice had a lower survival rate in infection, but a higher survival rate in sepsis as compared to their wild-type controls (unpublished data). Such a switch in the phenotype of plasminogen-deficient mice during bacterial infection and sepsis cannot be explained by the degradation of fibrin by plasmin. However, mice with a dysfunctional complement system show the same switch in the survival phenotype during bacterial infection and sepsis (Czermak et al., 1999; Cunnion et al., 2004). It therefore seems to be a functional correlation between plasmin formation and complement activation during non-autoimmune disease processes. Based on these findings and clues, I propose that there may be a strong functional correlation between plasmin and complement activation during inflammatory diseases. In order to describe this hypothesis better, in the following paragraphs I will summarize and compare the findings of the functional
roles of the PA and complement systems during different autoimmune and non-autoimmune disease models. In particular, I will focus on a comparison between the findings obtained in plasminogen-deficient mice and in mice deficient in components of the complement system.

Rheumatoid arthritis. Many lines of evidence support a key role for complement in the pathogenesis of inflammatory arthritis. For example, with anti-C5 monoclonal antibody treatment, mice with CIA have shown a marked diminution of inflammation and joint damage, suggesting a central role for complement in the pathogenesis of inflammatory arthritis (Wang et al., 1995). Genetic deficiency in the C5a receptor or in C5 expression completely protects mice from arthritis (Wang et al., 2000; Grant et al., 2002). In addition, mice deficient in C3 and factor B have been found to be resistant to CIA and passive antibody transfer model, suggesting that complement activation by both the classical and alternative pathways is critical for the effector phase of arthritis (Hietala et al., 2002; Hietala et al., 2004). Finally, initial studies using humanized mouse anti-C5 monoclonal antibodies in patients with RA have led to encouraging improvements, although an optimal therapeutic strategy for the disease is yet to be established (Kaplan, 2002). As shown in Papers I and II, plasminogen-deficient mice are resistant to CIA and CAIA. However, following a trauma administered to the knee joints, these mice become locally susceptible to CIA. The trauma initiates a wound-healing-like process which is not critically dependent on complement activation. Thus, it seems that plasmin probably plays an essential role in complement activation.

Multiple sclerosis. Although an increasing body of evidence suggests that complement is involved in the destruction of neurons and demyelination in MS (Hartung et al., 1992), disruption of the C3, C5, C3a receptor or C5a receptor genes fails to protect against development of EAE, especially the acute phase of the disease (Calida et al., 2001; Reiman et al., 2002; Weerth et al., 2003; Boos et al., 2004). Similarly, as shown in Paper III, plasminogen-deficient mice of B10.Q background developed similar levels of EAE and similar histopathological features of the spinal cords to wild-type controls.

Based on studies of plasminogen-deficient mice in the animal models for MS and RA, I propose a model describing the possible essential role of plasmin during the inflammatory cascade of autoimmune diseases (Figure 3). According to this model, it seems that plasmin only plays an essential role in those autoimmune diseases that are critically dependent on complement activation (Figure 3 and Papers I-III).

Wound healing. Studies using cobra venom factor to deplete serum complement showed that complement-depleted guinea pigs had marginally delayed infiltration of neutrophils in the first two days after incision wounds were performed, suggesting that the complement system is not the primary mediator of inflammation following an incision wound (Wahl et al., 1974). In another study, the development of dermal vascular damage in thermally injured rats was not affected by complement depletion, suggesting that the development of micro-vascular injury in dermal burn wounds is complement-independent (Ravage et al., 1998). Interestingly, plasminogen-deficient mice showed a delayed healing of incision wounds, and the inflammatory cell migration in general was not compromised (Romer et al., 1996). By reinvestigating this model, we showed that a big mass of necrotic tissue occurred under the epidermal layer of the skin in plasminogen-deficient mice, resulting in an impairment of adequate healing of the wounds. The wound closure was only slightly delayed and in general the inflammatory cell migration was not compromised.
Figure 3. Model for the possible functional role of plasmin during the inflammatory cascade of autoimmune RA and MS. CIA is suggested to be a B cell-mediated disease that is critically dependent on complement activation. After the induction of CIA, more than 83% of wild-type control mice developed arthritis, whereas plasminogen-deficient mice were completely resistant to development of disease. Antibody production following collagen type II immunization and also the deposition of labeled anti-collagen type II antibodies to the surface of cartilage were similar in wild-type and plasminogen-deficient mice. No signs of inflammation could be seen when plasminogen-deficient mice were induced with CAIA, and the disease could be restored after daily injections of human plasminogen in these mice. Thus, these results (as shown in Paper I) suggest that plasmin is essential for the induction of inflammatory joint destruction in CIA, most likely in the step of complement activation (shown with an asterisk). Injection of the knee joints with antigen or saline causes a trauma locally, which starts a wound-healing-like inflammatory response. It has been shown that the complement system does not play an essential role in wound healing. When such a trauma is administered to the knee joints in plasminogen-deficient mice after CIA induction, an inflammatory response with neutrophil infiltration is initiated, besides the autoimmune cascade against collagen type II. Accordingly, these plasminogen-deficient mice successfully developed mild local knee arthritis, although they were resistant to CIA. These results again confirmed that the resistance of the plasminogen-deficient mice to CIA is probably due to inability to activate the complement system in these mice (Paper II). EAE has generally been accepted to be a T cell-mediated autoimmune disease through Th1 cells. When MOG peptide 79-96 was used as the auto-antigen to induce EAE, plasminogen-deficient mice of B10.Q background developed levels of mild disease that were similar to those of wild-type littermates. Histopathology and immunostaining of spinal cords revealed that demyelination, and the amount of neutrophils and macrophages are similar in both plasminogen-deficient and wild-type mice (Paper III). Taken together, our studies on the role of plasmin in autoimmune diseases suggest that plasmin is essential for the autoimmune diseases that are critically dependent on complement activation. (manuscript in preparation). Previous studies have shown that necrosis occurs extensively in plasminogen-deficient mice in several wound healing models (Bezerra et al., 1999; Creemers et al., 2000; Drew et al., 2000b). The complement system has also been shown to be critical for clearance of necrosis/apoptotic tissue. In our studies of the healing of tympanic membrane perforations, inflammatory cell migration was not impaired in general. However, keratinocyte
migration was completely arrested in the plasminogen-deficient mice during the 143-day period after the perforation. The complete arrest of keratinocyte migration is probably due to necrosis occurring at the frontier of the perforation border (Paper IV). This was more evident in plasminogen-deficient mice after a dermal burn wound was induced (data not shown). A dermal burn wound causes immediate formation of necrosis at the dermal and subdermal parts of the skin. This necrotic tissue probably blocked the migration of dermal keratinocytes during the healing of the dermal wounds in plasminogen-deficient mice (manuscript in preparation). In summary, a working hypothesis is therefore that active plasmin plays its critical role in wound healing by interacting with the complement system to clear wounded area from necrotic tissue.

**Tissue and organ regeneration.** There are growing evidence suggesting that complement proteins not only serve as mediators of immune defense, but also as modulators of diverse developmental processes, such as cell survival, growth, and differentiation of various tissues (Mastellos and Lambris, 2002). In a recent study where it was shown that C3 and C5 are expressed in newt limb and lens regeneration, a link between complement biosynthesis and tissue regeneration was suggested (Kimura et al., 2003). These results have given rise to the concept that the complement system may have been selected through evolution as an important mediator of tissue regeneration, not only in lower vertebrates but also in more evolved species. Liver is one of the few quiescent organs in the adult body of mammals that has retained the ability to regenerate and restore its homeostasis in response to various perturbations (Michalopoulos and DeFrances, 1997). However, in C3 and C5-deficient mice, liver regeneration has been shown to be severely impaired after acute toxic injury of CCl4 (Mastellos et al., 2001; Strey et al., 2003; Markiewski et al., 2004). Interestingly, plasminogen-deficient mice also reveal an impairment of liver regeneration after toxic injury of CCl4 (Bezerra et al., 1999; Bezerra et al., 2001; Pohl et al., 2001). Although the authors of these studies did not propose any molecular mechanism for the impediment of the plasminogen-deficient mice from removing the necrotic tissue from a diseased hepatic microenvironment, they did observe that the subsequent reconstitution of normal liver architecture is in a fashion unrelated to circulating fibrinogen. With the plasmin-complement functional correlation hypothesis, the results obtained from the plasminogen-deficient mice in liver regeneration could be explained by the dysfunction of complement activation in these mice.

**Bacterial infection.** Bacterial pathogens can initiate the complement cascade, leading to signaling and inflammatory cell recruitment, opsonization, and formation of a membrane-attacking complex. Thus, the complement system is critical in host defense against bacteria in innate immunity. C3-deficient mice challenged with group B streptococci or with *Streptococcus pneumoniae* display a significant impairment of host defense (Wessels et al., 1995; Circolo et al., 1999). Mice deficient in C3 or C4, but not factor B, are more sensitive to the lethal effects of endotoxin than wild-type controls (Fischer et al., 1997; Matsumoto et al., 1997). Pulmonary mucosal host defense against *Pseudomonas aeruginosa* is impaired in C5a receptor-deficient mice, despite the fact that there is an increased pulmonary neutrophil infiltration in these mice as compared to wild-type controls (Hopken et al., 1996). The prevailing theory regarding the functional role of plasminogen in host defense against infection includes a role in inflammatory cell migration and interaction of the host plasminogen with endogenous PAs expressed on the surface of pathogens (Ploplis and Castellino, 2000; Ploplis, 2001). However, using this theory, many of our results as well as the findings of others cannot be fully explained. For instance, inflammatory cell migration in general is not impaired in plasminogen-deficient mice in chronic inflammation models (Romer et al., 1996). In our studies of bacterial arthritis, by intravenous
injection of *Staphylococcus aureus*, we have observed a significantly higher lethality in plasminogen-deficient mice than in wild-type controls. Furthermore, we found that the numbers of both neutrophils and bacteria that accumulate in the joints of plasminogen-deficient mice are much higher than in wild-type controls (unpublished data). These findings cannot be easily explained by previously proposed roles of plasmin host defense. However, they are in good accordance with the phenotypes obtained from complement-deficient mice.

**Sepsis.** Sepsis is defined as the systemic host response to an infection, which is often believed to be associated with the presence of bacteria in the blood (bacteremia). It has been suggested that patients are more endangered by the immune and inflammatory responses than by the invading microorganisms. In support of this suggestion, blockade of C5a generation with antibodies during the onset of sepsis has been shown to greatly improve survival in rodents (Czermak et al., 1999). Similar findings were made when the C5a receptor (C5aR) was blocked either by antibodies or by a small molecular inhibitor (Riedemann et al., 2002; Huber-Lang et al., 2002a). The mechanisms by which C5a exerts its harmful effects during sepsis are yet to be investigated in greater detail, but recent data suggest that generation of C5a during sepsis significantly compromises the innate immune functions of blood neutrophils (Huber-Lang et al., 2002b; Guo et al., 2003), including the ability to express a respiratory burst, and the ability to generate cytokines (Riedemann et al., 2003). In addition, C5a generation during sepsis appears to have procoagulant effects (Laudes et al., 2002). The concept of blockade of C5a/C5aR during sepsis therefore has a therapeutic potential, especially in the context of preventing sepsis development. In our infection studies, we found that after intravenous injection of a low number of bacteria, plasminogen-deficient mice have significantly higher mortality than wild-type controls. However, when mice were injected with a higher number of bacteria, death from sepsis was observed in more than half of the wild-type mice used, whereas death from sepsis was significantly delayed and decreased in plasminogen-deficient mice (data not shown). Such a switch in mortality phenotype in plasminogen-deficient mice during infection and sepsis is highly in line with studies of mice with dysfunction of the complement system, where the same switch in survival phenotype during bacterial infection and sepsis has been shown (Czermak et al., 1999; Cunnion et al., 2004). These phenotypic similarities suggest that there exist a functional correlation between the PA and complement systems during infection and sepsis. However, further studies are needed to prove this hypothesis and to elucidate the pathological pathways that link these two systems.

Taken together, the results obtained from our research and studies by other groups have suggested a strong functional correlation between plasmin and complement in various tissue remodeling processes. As discussed above, these processes include RA, MS, wound healing, tissue and organ regeneration, bacterial infection and sepsis, as well as tissue remodeling processes that are not included in the current discussion – such as mammalian reproduction, asthma, systemic lupus erythematosus, and bone and cartilage development. Investigations in order to prove or disprove this hypothesis will improve our understanding of the nature of the PA and complement systems and also the nature of the physiology involved.

10. **Summary of the present study**

This chapter is a summary of the five papers (numbered I-V) that this thesis is based upon. The figures and tables are referred to by their numbers as given in the original paper.
10.1. The plasminogen activator/plasmin system is essential for development of the joint inflammatory phase of collagen type II-induced arthritis (Paper I)

The PA system has been proposed to have important roles in RA. Studies of AIA have shown that uPA- and plasminogen-deficient mice have an exacerbated disease severity that correlates with the level of fibrin deposition. It has therefore been suggested that uPA and plasminogen may have major roles in fibrin removal in the AIA model. However, studies of uPA- and tPA-deficient mice of C57BL/6 genetic background in a CIA model showed that uPA-deficient mice develop only mild CIA, whereas tPA-deficient mice develop a more severe disease as compared to wild-type controls. We therefore set out to do an in-depth investigation of the functional roles of the PA system during CIA. In the study, we used uPA- and plasminogen-deficient mice with a CIA susceptible background (DBA/1).

CIA was first induced by collagen type II (CII) immunization in uPA-deficient, uPA-heterozygous and wild-type DBA/1 mice. Our data revealed that uPA-deficient mice have a lower severity and incidence of CIA than wild-type mice. Furthermore, while more than 80% of wild-type control mice developed CIA, none of fifty plasminogen-deficient littermates that were tested developed CIA within a forty-day period. In addition, the plasminogen-heterozygous mice, which have approximately 50% of the normal serum level of plasminogen, developed a lower severity and incidence of arthritis as compared to the wild-type control group (Table 2 and Figure 2, Paper I). Antibody generation following CII immunization as well as the binding of labeled anti-CII antibodies to the surface of cartilage, were similar in wild-type and plasminogen-deficient mice. No sign of inflammation could be seen when plasminogen-deficient mice were injected with a mixture of monoclonal antibodies against CII. However, after daily injections of human plasminogen these mice developed arthritis within 5 days.

The finding that infiltration of inflammatory cells into the synovial joints is impaired in plasminogen-deficient mice suggests that uPA and plasminogen are important mediators of joint inflammation. Active plasmin is therefore essential for the induction of pathologic inflammatory joint destruction in CIA, possibly in the step of complement activation.

10.2. Contrasting roles of plasminogen deficiency on arthritis in different rheumatoid arthritis models (Paper II)

AIA and CIA are two commonly used murine models of RA. AIA is induced by intradermal immunization and subsequent intra-articular injection of mBSA. The disease is chronic, antigen-specific, and T-cell dependent. CIA is induced by intradermal immunizations with the major cartilage protein component, CII. CIA is dependent on both B and T cells as well as the complement system. The pathogenic mechanisms of AIA and CIA differ in several respects including antigen challenge, disease induction and clinical features. Whereas AIA is a mono-arthritis model restricted to the locally injected knee joints, CIA is a poly-arthritis model in which the arthritis can develop in all the peripheral joints. The disease severity in CIA can be followed by a macro-scoring system, whereas the severity of AIA is evaluated by morphology.

In a previous study using the AIA model, it was shown that plasminogen-deficient mice develop much more severe arthritis than wild-type control mice (Busso et al., 1998). In contrast, our studies using the CIA model have shown that plasminogen-deficient mice are completely
resistant to CIA and we propose that plasmin may play its essential roles through activation of the complement system (Paper I). It is possible that the apparent discrepancy of the plasminogen-deficient phenotype during CIA and AIA could be explained by differences in the experimental set-up or the mouse strains used. However, more likely, the apparent discrepancy may reflect the different pathogenic mechanisms of these two arthritis models.

The aim of this study was to gain a deeper understanding of the molecular mechanism by which plasmin affects the pathogenesis of RA in the two different RA models. To do this, we have developed an alternative model denoted LIA, where CII or 0.9% NaCl is intra-articularly injected into the knee joints of previously CII-immunized mice. The difference between LIA and AIA is the antigen: CII is used in LIA and mBSA in AIA. The difference between LIA and CIA is the administration: a local trauma is induced in the knee joint in LIA, whereas there is no trauma in CIA. The local trauma will also initiate a wound-healing-like process in which the innate immune response against trauma is involved. By using these different models the different functional role of plasminogen in AIA and CIA can be interpreted and the mechanistic differences between the models can be elucidated more precisely.

By the use of the LIA model, we found that after CII immunization alone, wild-type mice developed arthritis in most of the paws as well as in the knee joints, whereas plasminogen-deficient mice were totally resistant to the disease. Local knee injections of CII or saline slightly enhanced the severity of the knee arthritis in wild-type mice. Unexpectedly also the plasminogen-deficient mice developed arthritis in joints that were injected with CII or saline, although the arthritis was milder than that in wild-type controls. Sustained tissue necrosis was found only in the plasminogen-deficient mice after the local injection. These results demonstrate that the trauma is critical to the disease profile of arthritis. Taken together, our data show that both the antigen and the joint trauma caused by the local injection can explain the difference in response between CIA and AIA models. This further indicates that CIA and AIA have distinct pathogenic mechanisms. The data also suggest that plasmin may be essentially required for the induction of arthritis in arthritis models that are critically dependent on complement activation.

10.3. Plasmin does not play an essential role in experimental autoimmune encephalomyelitis (Paper III)

The PA system has been suggested to play an important role in autoimmune inflammatory diseases, including RA and MS. Our studies have shown that plasminogen-deficient mice are resistant to autoimmune CIA, which indicates that plasmin is essential for the induction of CIA. EAE is the most commonly used animal model of MS. Here we used a myelin oligodendrocyte glycoprotein (MOG) peptide 79-96 as the immunogen, to examine the development of EAE in the absence or presence of the adjuvant pertussis toxin (PT) in plasminogen-deficient mice with two different genetic backgrounds.

When EAE was induced in the presence of PT, wild-type control mice of both B10.Q and a mixed (DBA/1 and C57BL/6) genetic backgrounds developed typical EAE, whereas most plasminogen-deficient mice developed EAE with an altered progression. The altered symptoms included tail spinning, hypersensitivity to disturbances, and lethality within hours. This result reveals a strong influence of PT on the plasminogen-deficient mice during the development of
EAE. When EAE was induced in the absence of PT, both wild-type and plasminogen-deficient mice of B10.Q background developed similar levels of mild disease with comparable levels of histopathological features, whereas both plasminogen-deficient mice and wild-type controls of a mixed (DBA/1 and C57BL/6) background were resistant to development of disease. In conclusion, this study indicates that plasmin is not essential for the development of EAE induced by MOG79-96. Furthermore, a strong influence of PT on plasminogen-deficient mice was observed during the development of EAE. Together with our previous findings on the role of plasmin in CIA, our data show that plasmin plays an essential and distinct role in development of arthritis but not for the development of encephalomyelitis.

10.4. Plasmin is essential for the healing of tympanic membrane perforations (Paper IV)

Wound healing is a dynamic tissue-interactive process involving inflammation, provisional matrix formation, angiogenesis, tissue formation and remodeling. TM perforations are commonly seen in clinical practice, e.g. after trauma to the ear or during episodes of acute otitis media. Previous studies of wound healing of skin and cornea as well as regeneration of several types of tissues after injury including myocardium, vasculature, liver and skeletal muscle have revealed that the phenotype of plasminogen-deficient mice can vary to a large extent depending on the model and the tissue involved. The healing of tympanic membrane perforations is a well-organized chain of inflammatory events, with an initial invasion of inflammatory cells followed by reparative and restoration phases. One important difference between the healing of TM perforations and the healing of skin wounds is the lack of provisional matrix formation. We therefore set out to study the role of plasminogen in the healing of TM perforations.

Perforations (standardized in size) were made in the posterior superior quadrant of the PT of the TM in wild-type control and plasminogen-deficient mice. Whereas the healing process in wild-type control mice was complete within 8-11 days after the perforation, the healing was totally arrested in plasminogen-deficient mice and there was no sign of healing even after 143 days. Several of the dynamic interactive processes associated with successful healing of the TM were disturbed in the plasminogen-deficient mice. Both neutrophils and macrophages were recruited to the wounded area, but the inflammatory response did not resolve as in wild-type mice. Moreover, there was no sign of the tissue debridement normally performed by these cells. In addition, the orchestration and execution of the subsequent steps of wound healing including removal of fibrin, keratinocyte migration and re-epithelialization, and in-growth of connective tissue were impaired in plasminogen-deficient mice.

The findings that re-epithelialization of the TM is completely blocked in plasminogen-deficient mice is in contrast to previous studies of skin and corneal wound healing, where re-epithelialization can occur in plasminogen-deficient mice. Plasmin therefore appears to play a more profound role in the healing of TM perforations than in other epithelial wounds. The data presented also suggest a new therapeutic strategy for curing certain types of TM perforations.

10.5. Spontaneous development of otitis media in plasminogen-deficient mice (Paper V)

Otitis media is defined as inflammatory conditions of the ear. The most important etiological factors related to otitis media are bacterial or viral infections of the upper respiratory tract.
Although several theories have been proposed, the exact etiology and pathogenesis of otitis media have not been conclusively established. An effective host defense during otitis media depends upon the integrated actions of inflammatory mediators and inflammatory cells. The PA system is a general proteolytic system thought to be involved in inflammatory cell migration, and also in the degradation of matrix proteins by leukocytes during host defense. During studies of the TM in plasminogen-deficient mice, we noticed that a number of plasminogen-deficient mice spontaneously developed chronic inflammatory changes of the middle ear under normal maintenance. In this study we examined the occurrence of spontaneous chronic otitis media in plasminogen-deficient mice. We followed the inflammatory response, and proposed possible mechanisms for the spontaneous development of chronic otitis media in these mice.

Six-week-old wild-type and plasminogen-deficient mice were selected. The status of the TM s and middle-ear cavities in the experimental mice was examined at the beginning of the experiment and at the ages of 9 weeks, 13 weeks, 18 weeks and 24 weeks. Whereas virtually all of the wild-type control mice kept a healthy status of the middle ear, all the plasminogen-deficient mice gradually developed chronic otitis media with various degrees of inflammatory changes during an 18-week observation period. Five bacterial strains were identified in materials obtained from the middle-ear cavities of six plasminogen-deficient mice. Morphological studies revealed the formation of an amorphous mass of tissue and inflammatory changes in the middle ears of plasminogen-deficient mice. Immunohistochemical studies further indicated a mass infiltration of neutrophils and macrophages, as well as the presence of T and B cells in the middle-ear mucosa of these mice. Extensive fibrin deposition and abnormal keratin formation were also observed in the tympanic membrane, the middle-ear cavity and the external ear canal of these mice.

These results suggest that plasminogen plays an essential role in protecting against the spontaneous development of chronic otitis media. Our findings also suggest the possibility of using plasminogen for clinical therapy of certain types of otitis media. In support of this, we have identified members of a family who suffer from chronic otitis media and recurrent TM perforations, with drastically reduced plasminogen levels (unpublished results).
CONCLUSIONS

- Plasmin formed by uPA is essential for the induction of inflammatory joint destruction in CIA.

- Plasmin plays contrasting roles in different models of rheumatoid arthritis.

- Plasmin is not required for the development of EAE induced by MOG79-96.

- Plasmin may only be essential in the autoimmune diseases that are critically dependent on complement activation.

- Plasmin appears to play a more profound role in the healing of TM perforations than in the healing of skin and corneal wounds.

- Plasmin plays an essential role in protecting against the spontaneous development of chronic otitis media.

- Taken together, plasmin appears to play essential roles during autoimmune and non-autoimmune diseases through a functional correlation with complement activation.
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