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URINARY TRACT INFECTION AND RENAL SCARRING

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Cover: Immunofluorescent staining of a section from the renal cortex of a mouse with pyelonephritis. The section is stained with propidium iodide (blue), neutrophil-specific antibody (red), and antibody to mouse cathelicidin CRAMP (green). The section represents the early stage of inflammation. Bacteria in the lumen of renal tubule stimulate the production and release of CRAMP. A neutrophil with typical segmented nucleus and positive for a neutrophil marker is also positive for CRAMP. For more details, please see paper I in this thesis.

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To my mum

‘…For it was you who formed my kidneys;
You knit me together in my mother’s womb.
I praise you, for I am fearfully and wonderfully made…’

Psalm 139, 13-14, King David, around 1000 BC

ABSTRACT

Urinary tract infection (UTI) is the most common bacterial infection. Although most patients with UTI have a good prognosis, there is a risk of serious complications in a group of them. In up to 40% of the cases of infection of the upper urinary tract, pyelonephritis, renal scar develops and the scarring process may occasionally lead to chronic renal insufficiency. Moreover, UTI has a high tendency of recurrence and recurrent UTIs even increase the risk of renal scarring.

We have been studying several mechanisms in the pathogenesis of UTI and renal scarring. We described the production of cathelicidin, an endogenous antibiotic, in the epithelial cells of the urinary tract. Although present in low concentrations constitutively, after bacterial attachment the epithelial cells rapidly increased the cathelicidin synthesis and release. Gene-deficient mice confirmed the relevance of the epithelial cathelicidin for the protection of the urinary tract against attaching bacteria.

Bacteria, however, seemed to develop strategies to overcome the 'protective shield' of cathelicidin. We showed that the majority of uropathogenic *E. coli* are able to produce curli fimbriae and cellulose and thus form a multicellular community, biofilm. This tactic helps bacteria adhere to the epithelium without stimulation of the immune system, invade the cells, and be protected from cathelicidin. The low levels of cathelicidin, on the other hand, inhibit the formation of biofilm, and protect the urinary tract also against this form of bacterial virulence.

Despite the effective antimicrobial defense of the urinary tract, bacteria sometimes win the 'first battle' against mucosal immunity and persist or invade the host. Then, more intense inflammation is needed with involvement of professional immune cells and with some inevitable degree of tissue damage. Cathelicidin is also an important player in these immune processes, in the recruitment of neutrophils and neutrophil-mediated killing of bacteria.

During inflammation in the kidney, many proteases and their inhibitors, amongst them MMP-9 and TIMP-1, are produced. Our data suggested that the delicate balance between MMP-9 and TIMP-1 determines the intensity of inflammation, degree of tissue destruction and proper healing after infection. If too high levels of TIMP-1 are produced during acute inflammation, renal scar will develop more probably. We indicated that the pro-scarring effect of TIMP-1 could be explained by its complex action during the acute inflammation. TIMP-1 prolongs the stay of granulocytes in the tissue by inhibiting their apoptosis and migration into urine. Moreover, TIMP-1 stimulates respiratory burst of neutrophils. 'Entrapped' and activated granulocytes may extensively destroy the tissue and trigger renal scarring.

Data presented in this thesis describe mechanisms in the pathogenesis of urinary tract infection and postinfective renal scarring. They also open new possibilities for treatment in order to protect the urinary tract from bacteria and the kidney from damage.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-IV):

I. Chromek M, Slamová Z, Bergman P, Kovács L, Podracká L, Ehrén I, Hökfelt T, Gudmundsson GH, Gallo RL, Agerberth B, Brauner A.
The antimicrobial peptide cathelicidin protects the urinary tract against invasive bacterial infection.
Nature Medicine. 2006 Jun;12(6):636-641

II. Chromek M, Wang X, Kádas L, Jacobson SH, Agerberth B, Römling U, Brauner A.
Uropathogenic *E. coli* produce biofilms to evade innate immune responses.
Manuscript.

III. Chromek M, Tullus K, Hertting O, Jaremko G, Khalil A, Li YH, Brauner A.
Matrix metalloproteinase-9 and tissue inhibitor of metalloproteinases-1 in acute pyelonephritis and renal scarring.
Pediatric Research. 2003 Apr;53(4):698-705

IV. Chromek M, Tullus K, Lundahl J, Brauner A.
Tissue inhibitor of metalloproteinase 1 activates normal human granulocytes, protects them from apoptosis, and blocks their transmigration during inflammation.
Infection and Immunity. 2004 Jan;72(1):82-88

CONTENTS

ABBREVIATIONS

AV	Annexin V
CAMP	Gene for human cathelicidin LL-37/hCAP-18
Camp	Gene for mouse cathelicidin CRAMP
CLED	Cystein lactose electrolyte deficient
CNF-1	Cytotoxic necrotizing factor-1
CRAMP	Cathelicidin related antimicrobial peptide
CXCR1	CXC chemokine receptor 1, known also as interleukin-8 receptor 1
DCFH	Dichlorofluorescein diacetate
DMSA	^{99m}Tc-dimercaptosuccinic acid
E. coli	*Escherichia coli*
EC_{50}	50% effective bactericidal concentration
ECM	Extracellular matrix
EDTA	Ethylene-diamine-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
hCAP-18	Human cationic antimicrobial protein-18, precursor of LL-37
IL	Interleukin
LB	Luria-Bertani
LL-37	Active human cathelicidin peptide
LPS	Lipopolysaccharide
MIC	Minimal inhibitory concentration
MMP	Matrix metalloproteinase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of metalloproteinases
TNF-α	Tumor-necrotizing factor-α
TLR	Toll-like receptor
UTI	Urinary tract infection
VUR	Vesico-ureteral reflux

INTRODUCTION

Urinary tract infection (UTI) is considered to be the most common bacterial infection (Foxman 2002) and affects mainly children and women. The cumulative incidence rate in children reaches 10%. In adulthood, almost half of all women will experience at least one infection during their lifetime (Craig 2001; Foxman 2002; Marild et al. 1998). UTI is classified according to the dominant site of infection into infection of the lower urinary tract, cystitis and infection of the upper urinary tract, pyelonephritis. When bacteria reach the blood stream, the disease may cause septicemia.

Although most patients with UTIs have an excellent prognosis, there is a risk of complications in a group of them. Renal scarring develops in 10-40% of the cases of pyelonephritis, depending on which method is used to detect the scar; renal scintigraphy (Stokland et al. 1996), urography (Stokland et al. 1998), computerized tomography (Saxton 1995) or magnetic resonance imaging (Kavanagh et al. 2004). The clinical relevance of all detected scars has been widely debated. Renal scars have been correlated with long-term problems, such as hypertension (Jacobson et al. 1992), pregnancy complications (Martinell et al. 1990), and in the most severe cases end-stage renal disease. Other studies, in contrast, indicate that the risk could be overestimated (Wennerstrom et al. 2000a; Wennerstrom et al. 2000b) and that infection *per se* does not cause renal scarring (Sreenarasimhaiah et al. 1998). Nevertheless, from a global view, urinary tract infection is still one of the main causes leading to end-stage renal disease mainly in children (van der Heijden et al. 2004).

Urinary tract infection also carries a high risk of recurrence. Approximately 30% to 40% of patients develop a repeat infection within one year after a first UTI (Foxman 2002; Winberg et al. 1974). This rate becomes even higher after subsequent infections (Schlager et al. 1993). In order to identify individuals at risk of recurrence, different radiological tests have been introduced after a first pyelonephritis (Hoberman et al. 2003). Still, more than one half of the patients have normal radiographic studies (Jodal 1987), and also these patients have high probability to develop consequent infections (Mingin et al. 2004).

UTI remains a big problem for the patients, their families and health care professionals. There are important financial implications associated with UTI, mainly

because of multiple medical visits, antimicrobial prescriptions, hospitalization expenses, as well as costs connected with travel and sick days (Foxman 2002).

The urinary tract infection involves multiple interactions between the bacterial pathogen and host. Although difficult to separate from each other, the bacterial and host factors will be described separately.

PATHOGENESIS OF UTI

Bacterial factors

Escherichia coli (E. coli) is the most commonly isolated pathogen, both in community-acquired and hospitalized patient UTI (Farrell et al. 2003; Gruneberg 1994; Ronald 2002), followed by Klebsiella, Enterobacter, Proteus species, enterococci, and among community-acquired UTI also *Staphylococcus saprophyticus* (Ronald 2003).

UTI is thought to begin when a bacterial clone from the intestinal flora colonizes the periurethra and then ascends into the bladder. *E. coli* typically colonizes the gastrointestinal tract of humans already within few hours after birth. Some well established *E. coli* clones can acquire specific virulence attributes with increased ability to adapt to new niches (Kaper et al. 2004). These virulence properties are frequently encoded in specific genetic elements, so called 'pathogenicity islands'. Only the most successful combination of virulence factors has persisted to form a specific type, uropathogenic *E. coli*. The subset of *E. coli* that causes uncomplicated cystitis and acute pyelonephritis is distinct from the commensal *E. coli* strains. These phenotypic characteristics include specific adhesins, toxins, siderophores, proteases and the capsule, as well as hydrophobicity and serum resistance (Kaper et al. 2004; Manges et al. 2001; Snyder et al. 2004; Tullus et al. 1991). Despite some common features, there is no single profile that would cause UTIs. Uropathogenic *E. coli* uses a multi-step scheme of pathogenesis, which consists of adhesion and colonization, invasion, survival, multiplication and host damage (Kaper et al. 2004). Accordingly, bacterial virulence factors could be divided into adhesion/colonization factors, survival/immune escape factors, and toxins.

Adhesion and colonization

Pathogenic *E. coli* possess specific adherence factors that allow them to colonize different sites that *E. coli* do not normally inhabit. Adhesion to the cells could be a

function of physicochemical surface properties of bacteria as determined by a specific composition of lipopolysaccharides (LPS) and the capsule (Ohman et al. 1981; Tullus et al. 1991) and often described as hydrophobicity. The saccharide part of LPS is usually referred to as O-antigen. Interestingly, only a small number of O-serogroups has phenotypes that are epidemiologically associated with urinary tract infection (Johnson et al. 1994; Orskov et al. 1982).

In addition to LPS and the capsule, pathogenic *E. coli* express a number of distinct adhesins. Adhesins usually form definite morphological structures called fimbriae (also called pili) and fibrille. Fimbriae are rod-like structures of 5-10 nm in diameter. Fibrille, on the other hand, are only 2-4 nm in diameter, and are either long and wiry or curly and flexible (Kaper et al. 2004). Uropathogenic *E. coli* is characterized by type 1 fimbriae, P fimbriae, S fimbriae, F1C fimbriae and Dr adhesins (Guyer et al. 2001; Mulvey 2002). The expression of different fimbriae during UTI seems to be a coordinated process and *E. coli* express mainly one fimbrial type at a time (Nowicki et al. 1984; Snyder et al. 2005). In early stages of urinary tract infection, type 1 fimbriae seem to be important. Type 1 fimbriated *E. coli* attach to mannose moieties of the uroplakin receptors that coat transitional uroepithelial cells (Bahrani-Mougeot et al. 2002; Connell et al. 1996; Gunther et al. 2001). In strains that cause cystitis, type 1 fimbriae are continually expressed and the infection is confined to the bladder (Connell et al. 1996). In pyelonephritic strains, on the other hand, the type 1 fimbriae expression turns 'off' (Gunther et al. 2001). This may allow the organism to ascend through the ureters to the kidneys, where the bacterium can attach by P fimbriae to digalactoside receptors that are expressed on the kidney epithelium (Dodson et al. 2001; Soderhall et al. 1997; Svanborg-Eden et al. 1978). S fimbriae and F1C fimbriae have also been shown to bind to epithelial and endothelial cells from the kidney and lower urinary tract (Emody et al. 2003; Marre et al. 1990). Dr fimbriae, on the other hand, bind to type IV collagen and decay-accelerating factor, and enable *E. coli* to persist longer in the renal interstitium (Selvarangan et al. 2004). Curli fimbriae belong to a class of fibers known as amyloids (Barnhart et al. 2006). They may play a role in adherence and internalization by epithelial cells but the *in vivo* relevance of curli is still not clear (Gophna et al. 2001; Kikuchi et al. 2005).

Some uropathogenic *E. coli* strains have been shown to express flagella. Flagella are up to 15 μm long complex organelles, which contribute to bacterial mobility (Ramos et al. 2004). Flagellar motility and swarm cell differentiation contribute to the

virulence of another urinary pathogen, *Proteus mirabilis* (Allison et al. 1994; Harmon et al. 1989). In contrast, the role of flagella in the colonization of urinary tract by *E. coli* seems to be of a subordinate importance (Wright et al. 2005; Yamamoto et al. 1990).

Survival and immune escape

Pathogenic *E. coli* have developed several strategies to survive in an environment, which is sometimes not very favorable and poor of nutrients. Amongst these protective factors, siderophores, capsule and biofilm appear to be the most important. Aerobactin, an example of a siderophore, enhances iron uptake and thus promote the survival and growth of bacteria within the urinary tract (Jacobson et al. 1988). *E. coli* is able to produce a polysaccharide capsule, which substantially increases bacterial survival within the urinary tract and increases resistance to serum and to phagocytosis (Bahrani-Mougeot et al. 2002; Cross et al. 1984; Mellata et al. 2003). In the presence of indwelling catheters, bacteria form multicellular communities, biofilms, which protect them from attacks of the immune system and exogenous antibiotics (Trautner et al. 2004). It is still not clear if biofilm also plays a role in community-acquired UTI.

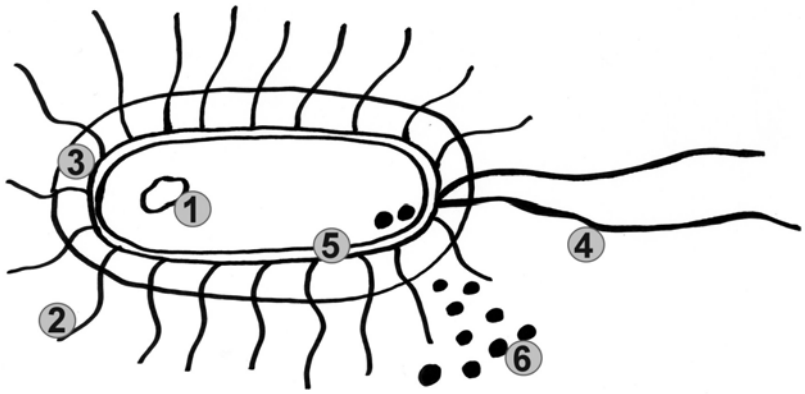

Figure 1. *Uropathogenic E. coli*: (1) Bacterial chromosome with pathogenicity island (-s), (2) Fimbriae, (3) Capsule, (4) Flagella, (5) LPS as a constituent of cytoplasmatic membrane, (6) Secreted toxins

Toxins

E. coli produce a number of toxins, which are either associated with the membrane or secreted. Toxins participate in the pathogenesis of urinary tract infection by different mechanisms. LPS, bacterial endotoxin, a principal component of the bacterial cell membrane is recognized by the immune system as a pathogen associated molecular pattern and initiates local and systemic response. Its toxicity relates to the side effects of triggered immune reaction (Alexander et al. 2001). Secreted hemolysin can,

depending on the concentration, induce distinct oscillations of calcium concentration in renal epithelial cells (Uhlen et al. 2000) or even cell necrosis. The mechanisms of action of cytotoxic necrotizing factor-1 (CNF-1) involve the Rho-dependent rearrangement of the cytoskeleton in eukaryotic cells with a complex of consequences. CNF-1 *in vitro* influences apoptosis (Fiorentini et al. 1998; Mills et al. 2000), phagocytosis (Hofman et al. 2000) and enhances secretion of proinflammatory cytokines by different cell types (Falzano et al. 2003). However, there are conflicting data about the role of CNF-1 *in vivo* (Johnson et al. 2000; Rippere-Lampe et al. 2001). Secretion of another toxin, Sat damages glomeruli and causes vacuolization in the surrounding epithelium (Guyer et al. 2000). Also Pic and Tsh proteases have been shown to be associated with uropathogenic and not fecal *E. coli* strains although their role in pathogenesis of UTI is still not clear (Heimer et al. 2004).

Host factors

The urinary tract is one of the exclusive areas of the body, which normally resists microbes' growth despite its close proximity to the outside environment and frequent bacterial entry (Hooton et al. 2000a). Various factors are involved in bacterial clearance, both constitutive and those inducible by the presence of a pathogen.

Constitutive host mechanisms

Constitutive mechanisms involved in the clearance of bacteria include mechanical factors such as urine flow and regular bladder emptying. Moreover, chemical defense components of epithelia seem to be important. Tamm-Horsfall protein blocks the adherence of type 1 piliated *E. coli* to uroplakin receptors (Bates et al. 2004; Serafini-Cessi et al. 2003). Secretory IgA (Bueler et al. 1995; Fliedner et al. 1986) and low molecular weight sugars (Mulvey et al. 2000) can also block the adherence of uropathogens. Lactoferrin (Abrink et al. 2000) inhibits the growth of bacteria by decreasing the accessibility of iron, and antimicrobial peptides directly kill bacteria by destroying their membrane (Bals et al. 1998; Valore et al. 1998).

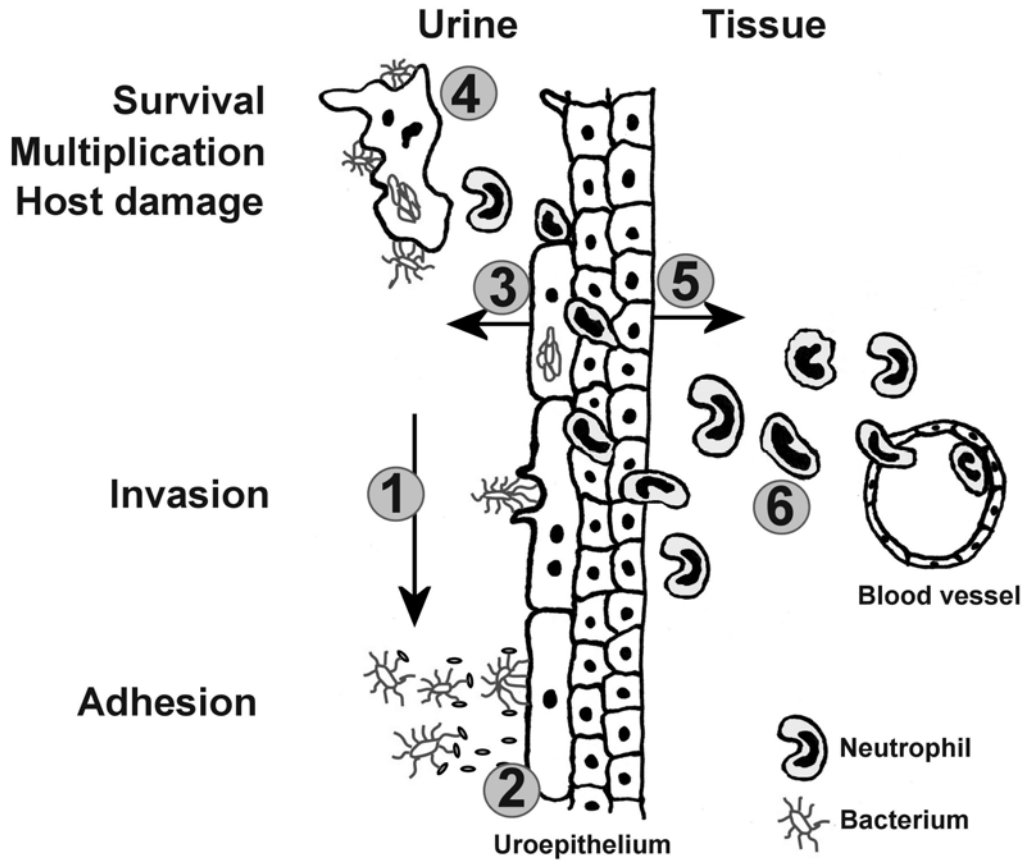

Figure 2. *Host factors in the protection against bacteria.* (1) Urine flow, (2) Antiadherence factors (e.g. Tamm-Horsfall protein), (3) Antimicrobial factors (e.g. nitric oxide, antimicrobial peptides), (4) Exfoliation of cells, (5) Production of chemokines and cytokines, (6) Migration of neutrophils. The ratio in size between bacteria and human cells does not correspond to reality.

Inducible host mechanisms

The presence of bacterium induces robust immune response already after a short contact with uroepithelial cells. Bladder superficial epithelial cells express toll-like receptor-4 (TLR-4) on their membrane (Backhed et al. 2001; Samuelsson et al. 2004), which, together with its co-receptors CD-14 and MD-2, recognizes LPS from bacteria and activates innate immune response. There are conflicting results regarding the expression of CD-14 on uroepithelial cells. Several studies showed the presence of CD-14 on the protein and mRNA levels (Backhed et al. 2001; Chromek et al. 2005; Schilling et al. 2003). Other groups, in contrast, did not detect CD-14 expression on uroepithelial cells (Samuelsson et al. 2004) suggesting that the response of uroepithelial cells to LPS requires the presence of type-1 (Hedlund et al. 2001) or P-fimbriae (Frendeus et al. 2001b). The latter mechanism could explain the strong immune response to virulent strains expressing fimbriae, which is absent in the presence of non-virulent strains causing asymptomatic bacteriuria (Bergsten et al. 2004; Eden et al.

1976). Alternatively, urinary soluble CD-14 was proposed to co-mediate LPS-signaling although it remains unclear if its urinary levels in health are high enough for such an effect (Bussolati et al. 2002). In addition to TLR-4, TLR-11 has been implicated as an important player in the defense of the host against UTI in mice. However, the molecule recognized by TLR-11 is currently unknown and its functional relevance for the human UTI remains questionable (Zhang et al. 2004).

During pyelonephritis, renal tubular epithelial cells are the main cells to react to the presence of bacteria (Brauner et al. 2001). The mechanism of sensing bacteria has also here been a matter of debate and conflicting results. Some studies showed that renal tubular cells did not express CD14 or TLR-4, and were hyporesponsive to LPS stimulation (Backhed et al. 2001; Schilling et al. 2003). Others, in contrast, were able to detect TLR-4 on renal tubular epithelial cells (Hedlund et al. 2001; Samuelsson et al. 2004). Despite the described controversies, epithelial cells within the urinary tract appear to be not just a mechanical barrier but also an active part of the innate immune system sensing bacteria and triggering the cascade of immune response to them (Chowdhury et al. 2004).

After contact with bacteria, epithelial cells react by different ways. They produce substances toxic to bacteria, like nitric oxide (Poljakovic et al. 2001) and β-defensin-1 (Valore et al. 1998). In addition, bladder epithelium exfoliates superficial facet cells (Mulvey et al. 2000), which helps clearing the bacteria from the bladder. Epithelium can also engage other cells by production of chemokines and proinflammatory cytokines (Khalil et al. 1997). Chemokines attract professional immune system cells, and cytokines activate them. Out of a number of chemokines and proinflammatory cytokines, interleukin 8 (IL-8) seems to be of crucial importance (Godaly et al. 1997) because of its chemoattraction of neutrophils (Haraoka et al. 1999). Cytokine-mediated up regulation of adhesion molecules and cytokine receptors facilitates the process of migration of immune cells. Amongst them, CXCR1 receptor on renal epithelial cells has been shown to be of importance for transepithelial migration of neutrophil granulocytes and bacterial clearance during urinary tract infection (Frendeus et al. 2000). Neutrophils kill bacteria by different mechanisms, either phagocytosis or the release of the toxic content of their granules (Lee et al. 2003). The influx of neutrophils is followed by an influx of other professional immune cells, namely monocytes/macrophages and lymphocytes, which are predominantly important in later stages of infection.

Contribution of the adaptive immunity to the protection of the urinary tract against infection has also been extensively studied. Mice with deficiencies in T and B lymphocyte function remained equally resistant to infection as their immunocompetent counterparts (Frendeus et al. 2001a; Svanborg Eden et al. 1984). Similarly, patients with deficiencies in the adaptive immune defenses are not known to have higher susceptibility to UTI. Adaptive immune system does therefore not seem to play a primary role in the protection of the urinary tract against bacteria.

PATHOGENESIS OF RENAL SCARRING

Interstitial fibrosis is the final common end-point of progressive renal diseases. Despite its clinical significance, the mechanisms of scar formation in the kidney are still not fully understood. The risk of renal scarring increases with each new episode of UTI. Factors predisposing for recurrence of UTI are therefore also risk factors of renal scarring.

Bacterial factors

Different bacterial virulence factors have been suggested to directly trigger the scarring process in the kidney. The presence of P fimbriae was proposed as a risk for renal scarring in some studies (Winberg et al. 1982). Others found no difference in the frequency of P-fimbriated *E. coli* (Jacobson et al. 1985; Jantausch et al. 1992) or even showed significantly lower frequency of P fimbriated *E. coli* in children developing renal scars as compared with those without scarring (Lomberg et al. 1989). One group also suggested type 1 fimbriae as a causative agent in the pathogenesis in postinfective renal scarring process (Topley et al. 1989), which was not confirmed by other studies.

Although bacterial virulence factors do not seem to trigger scarring directly, they may be important for the development of persistent or recurrent UTI. Studies using mouse UTI model systems and cell cultures demonstrated that *E.* coli can invade bladder superficial cells (Anderson et al. 2004; Anderson et al. 2003). Invasion of host cells by uropathogenic *E. coli* is promoted by distinct virulence factors, including cytotoxic necrotizing factor (Chung et al. 2003), Afa/Dr adhesins (Goluszko et al. 1997), type 1 fimbriae (Bower et al. 2005), and curli fimbriae (Gophna et al. 2001). As the urinary bladder fills and empties, extensive exocytic and endocytic traffic of

membrane domains of epithelial cells (Apodaca 2004) may facilitate entry of bacteria bound to membrane uroplakins. Intracellularly, bacteria become embedded in polysaccharide- rich matrix surrounded by a protective shell of uroplakin (Anderson et al. 2003). Estimated half-life of uroepithelial cells is 3-6 months (Apodaca 2004), which may promote the persistence of intracellular bacteria in the urinary tract. By this mechanism, bacteria may survive and cause a relapse of urinary tract infection (Justice et al. 2004). In the presence of foreign body in the urinary tract, e.g. catheter, bacteria may also form biofilm, an important source of infection (Trautner & Darouiche 2004).

Host factors

Anatomical anomalies of the urinary tract

Anatomical obstruction (posterior urethral valves, ureteropelvic junction obstruction, ureterovesical obstruction, and ureterocele) is seen in 2-10% cases of UTI (Spencer et al. 1986). Renal scarring could be induced by obstruction per se (Chevalier 2004) or it could be a result of accompanied urinary tract infections (Hooton 2000).

The most common urological anomaly in children is vesico-ureteral reflux (VUR) and as such it has been a matter of considerable concern. VUR can be secondary, for example as a consequence of anatomical obstruction of urine flow. More often, it is a primary condition associated with a failure of the maturation of uretero-vesical valve structure and function. VUR is found in 1-2% of all children but in 25-40% of children with acute pyelonephritis (Fanos et al. 2004). It is closely associated with UTI and renal scarring. There have been several attempts to prevent such kidney damage by different treatment strategies, such as surgical correction of the VUR or by long-term antibiotic prophylaxis of UTI. However, the results of both approaches remain questionable (Fanos & Cataldi 2004; Garin et al. 2006; Wheeler et al. 2004) and do not seem to improve the outcome as measured by kidney damage. It appears that primary VUR may be just a marker for generalized disease of the whole urinary tract, which includes dysplasia or hypoplasia, altered urinary bladder function, and perhaps a predisposition to UTI which is not cured when VUR regresses or is surgically corrected (Fanos & Cataldi 2004; Gordon et al. 2003; Hellerstein 2000; Wennerstrom et al. 2000c).

Dysfunctional elimination

Functional abnormalities of the lower urinary tract or dysfunctional elimination syndrome denote a group of abnormal voiding patterns, such as bladder instability, infrequent voiding, lazy bladder and the Hinman syndrome (Koff et al. 1998). Voiding disturbances could influence colonization of perineum, the entry of uropathogens (vagino-urethral reflux) or intravesical growth of bacteria. Voiding dysfunction was clearly associated with recurrent UTIs (Bakker et al. 2004) and complex treatment of the dysfunction resulted in a substantial decrease of the frequency of UTI attacks (Saedi et al. 2003). The clear association between VUR and dysfunctional elimination syndrome in children may also explain in part, the relationship between VUR, recurrent UTI and renal scarring (Greenfield et al. 2000; Koff et al. 1998; Mingin et al. 2004; Snodgrass 1998).

Immune response during acute pyelonephritis

Several studies have shown a correlation between a type of cytokine profile during the acute phase of pyelonephritis and development of renal scar. Clinical observations have suggested a link between increased urinary levels of interleukin-6, acute kidney damage (Roilides et al. 1999), and postinfective renal scar development (Tullus et al. 1994; Wang et al. 2001). On the other hand, persisting high urinary levels of IL-1α were associated with less inflammation and scarring (Tullus et al. 1996).

The tissue damage by polymorphonuclear leukocytes and macrophages seems to be of key importance in the initiation of renal scarring. Both blocking of polymorphonuclear leukocytes chemoattraction and migration resulted in excessive scarring (Hang et al. 2000; Svanborg et al. 2006). Reduction of the release or activity of toxic products from neutrophils, in accordance, led to the reduction of postinfective fibrosis (Bille et al. 1982; Huang et al. 1999; Matsumoto et al. 1990; Pohl et al. 1999; Roberts et al. 1986).

Balance between the degree of cell proliferation and deletion, either by necrosis or apoptosis, determines the cell number in the kidney. During acute pyelonephritis, many resident renal cells die as a result of toxic effect of bacteria as well as due to hypoperfusion and tissue hypoxia (Jahnukainen et al. 2005; Trifillis et al. 1994). Acute phase of inflammation is followed by a reperfusion period characterized by a production of free oxygen radicals by tubular cells. Thus, oxygen radicals originating from both immune cells and tubular epithelium may participate in tissue injury during

acute inflammation and reperfusion (Jahnukainen et al. 2005; Okur et al. 2003). Typically, compensatory hypertrophy and hyperplasia of renal cells follow after early stages of renal injury. For the resolution of inflammation, efficient apoptosis of excessive and infiltrating cells is important. Progressive apoptosis of renal cells, on the other hand, has been implicated in the renal scarring process (Thomas et al. 1998; Truong et al. 1996).

Mainly in non-infectious causes of renal fibrosis, a connection with growth factors such as transforming growth factor beta (TGF-β1), platelet derived growth factor, and connective tissue growth factor have been suggested (Bonner 2004; Khalil et al. 2000b; Okada et al. 2005; Sharma et al. 1994). Also, endogenous antifibrotic factors have been identified, particularly hepatocyte growth factor, bone morphogenetic protein-7, Smad transcriptional corepressors (Liu 2004; Liu 2006; Yang et al. 2003) and relaxin (Heeg et al. 2005; Samuel et al. 2006).

Normal tissue healing appears to be a result of a balance between extracellular matrix (ECM) protein synthesis and degradation (Eddy 2000). Acute phase of inflammation is characterized by an increased ECM production and reduced matrix degradation, which results in a deposition of extracellular matrix components, mainly collagen. The pro-fibrotic state could be a consequence of excessive or prolonged increase of ECM protein synthesis and/or a decrease of proteolytic degradation with accumulation of collagen (Gonzalez-Avila et al. 1998). Inhibitors of proteolysis, namely plasminogen activator inhibitor 1 and tissue inhibitor of metalloproteinases 1 (TIMP-1) have been proposed as major elements in this process (Eddy 2002). Accumulated collagen is cross-linked by the tissue transglutaminase, which promotes the process of scarring by several mechanisms, matrix stabilization, activation of TGF-β1, and rescuing fibroblasts from apoptosis (Verderio et al. 2004).

Many studies have also shown a connection between the renin-angiotensin-aldosterone system and renal fibrosis (Brown et al. 2002; Hollenberg 2004). Both angiotensin and aldosterone can influence different components of the renal scarring process. Blocking their effect could prevent or even induce regression of renal fibrosis (Fogo 2003; Khalil et al. 2000a; Miyata et al. 2006).

DNA polymorphism

The role of several genetic polymorphisms has been studied in the pathogenesis of renal scarring, e.g. polymorphisms influencing the levels and activity of

proinflammatory cytokines and their receptors, adhesion molecules, growth factors, polymorphisms in the renin-angiotesin-aldosteron system, and in the genes for enzymes involved in ECM remodeling (Ishihara et al. 2002; Pardo et al. 2003; Witkin et al. 2002; Ye 2000). Out of the described polymorphisms, genetic variability in the gene for intercellular adhesion molecule-1 (Gbadegesin et al. 2006) and TGF-β1 (Cotton et al. 2002; Lee-Chen et al. 2004) have been related to the presence of renal parenchymal scarring following UTI.

Factors possibly associated with recurrent UTIs and renal scarring

As risk factors for recurrent UTIs in women, sexual activity and the use of contraception (Hooton 2001; Scholes et al. 2000) have been well established. Also, several components of the mucosal immunity of the urinary tract have been investigated. In some studies, secretory IgA (Fliedner et al. 1986) and neutrophil function (Condron et al. 2003) have been shown to be affected in girls with recurrent UTIs.

In connection with recurrent UTIs in boys, circumcision is extensively discussed in the literature. Although done very often, this procedure as a measure to reduce recurrent UTIs remains controversial (Poland 1990; Singh-Grewal et al. 2005; To et al. 1998; Williams et al. 1993).

The age-related susceptibility of renal parenchyma seems likely but has not been conclusively confirmed. Several studies reported that the susceptibility of the renal parenchyma to infection and postinfective damage varies according to the age, with infants under 1 year at the greater risk (Martinell et al. 1995; Pylkkanen et al. 1981). This general belief was, however, challenged by other prospective studies using DMSA scintigraphy (Benador et al. 1997; Orellana et al. 2004).

Some studies have also suggested that early diagnosis of pyelonephritis with early and appropriate antibiotic treatment is critical to preserve renal function and to protect the kidney from damage (Slotki et al. 1982; Smellie et al. 1994).

AIMS OF THE THESIS

The general aim of this thesis was to elucidate factors in the pathogenesis of urinary tract infection and renal scarring.

The specific aims were to study:

I. The expression of the antimicrobial peptide cathelicidin in the urinary tract and its role in the protection of the urinary tract against infection.

II. The production of biofilm by *E. coli* from community-acquired UTI and its role in the pathogenesis of UTI.

III. The expression and role of MMP-9 and TIMP-1 in pyelonephritis and in renal scarring.

IV. The mechanisms how TIMP-1 promotes the scarring process during acute inflammation.

MATERIAL AND METHODS

CLINICAL STUDIES

We carried out two clinical studies involving children (papers I and II, and papers III and IV, respectively) and one clinical study involving adult women suffering from urinary tract infection (paper II). The diagnostic criterion of acute UTI in the studies was the presence of $\geq 10^5$ of *E. coli* per ml of freshly voided urine. In addition to bacteriuria, the diagnostic criteria of acute pyelonephritis were: body temperature ≥ 38°C and laboratory signs of systemic inflammation, either C-reactive protein ≥ 20 mg/l or erythrocyte sedimentation rate ≥ 20 mm/hr. We isolated bacterial strains from urine of all patients. From children, we analyzed urine samples during the acute phase of the disease and after 6 weeks. ^{99m}Tc-dimercaptosuccinic acid scintigraphy (DMSA) was performed within 10 days after admission to the hospital and 1 year after the acute infection. This method has been shown to detect acute renal damage and renal scarring with high sensitivity and specificity (Shanon et al. 1992). From the size and degree of a defect in DMSA uptake, a DMSA score was calculated as described before (Tullus et al. 1994). The number of leukocytes in urine was determined by microscopic evaluation of urinary sediment (paper I) or semi-quantitatively (paper III and IV).

As a control, we analyzed urine samples from a group of children without any signs of inflammation or renal disease (papers I and III). *E. coli* commensal strains isolated in connection with routine examination have been used as a control in paper II.

Healthy tissue from different parts of the kidney of patients nephrectomized due to a localized malignant tumor affecting the kidney has been used to identify the local production of cathelicidin in health or after infection of tissue pieces *in vitro* (paper I). We also isolated proximal tubular cells from the renal cortex for the *in vitro* experiments (paper I). In order to study the functions of granulocytes *in vitro*, we prepared granulocytes from healthy volunteers (paper IV).

ANIMAL EXPERIMENTS

A mouse model of acute ascendant pyelonephritis has been used. In the majority of experiments, we used the outbred mouse strain NMRI (papers I and III). To evaluate

the relevance of the cathelicidin production for the protection of the urinary tract, CRAMP-deficient, *Camp*$^{-/-}$ mice were infected and compared with parental strains, C57Bl/6 and 129/SvJ mice (paper I).

In all the animal experiments, we catheterized the animals under anesthesia and instilled bacterial suspension into the urinary bladder. In experiments presented in paper III, after bacterial instillation, a 6-h obstruction of the urethra has been performed. At preset time points, the animals were euthanized. Urinary bladders and kidneys where then analyzed for the presence of bacteria, as well as the organs were taken for the ELISA and immunohistochemistry analysis. In order to study the impact of neutrophils for the course of UTI, we induced short-term neutropenia in animals by intravenous treatment with neutrophil-specific monoclonal antibody 24 h prior infection (paper I).

IN VITRO EXPERIMENTS

Cells

For the *in vitro* studies, we used both immortalized cell lines and primary cells. As a model of uroepithelium, the first epithelium being in contact with bacteria during UTI, we employed the cell line J82 derived from a cancer of the urinary bladder (papers I and III), and the virus-immortalized cell line UROtsa (papers I, II and III). In order to mimic the inflammation in the kidney, we isolated proximal tubular cells from human renal tissue as well as from mouse kidneys (paper I). Additionally, we cultured renal epithelial cell line A498 derived from a cancer of the kidney (all papers). For paper III, we also cultured normal human mesangial cells representing mesenchymal cells of the kidney.

Bacteria

For the majority of animal experiments as well as *in vitro* studies, well-characterized uropathogenic strain *E. coli* CFTO73 has been used (papers I, III and IV). For the biofilm project (paper II), we chose from the clinical study a typical uropathogenic *E. coli* strain No 12, producing biofilm. By knocking the structural genes for the cellulose and/or curli, we constructed following mutants: WE1 *bcsA*::Cm, deficient in cellulose production, WE10.01 *csgD bcsA*::Cm, deficient in both cellulose production and curli expression, and WE11.01 *csgBA*::Cm, deficient in curli expression. Before the experiments, bacteria were grown under different conditions. To promote the growth in

a planktonic phase, bacteria were first cultured overnight on cystein lactose electrolyte deficient (CLED) agar plates at 37°C and then, to reach the logarithmic phase of growth, in LB broth in a horizontal shaker for 4 h. To prepare bacteria in a phase of broken biofilm, bacteria were first grown overnight on LB agar plates without salt at 37°C. Colonies were then scrapped off the plates and suspended in PBS.

Detection of biofilm formation by bacteria

We measured the adhesion to polystyrene and the thickness of biofilm by the microtiter plate method to screen clinical isolates of *E. coli* and to test the effect of synthetic LL-37 on the biofilm formation. Bacteria grew in 96-well plates in LB broth without salt at 37°C for 24 h. After incubation, the content of each well was gently aspirated by tipping the plate and washed with PBS. The plates were then air-dried to fix adherent bacteria and stained with crystal violet solution. After washing and solubilization of the bound dye with ethanol, the optical density was measured. In order to analyze the composition of extracellular matrix of bacterial biofilm, bacteria were cultured on Congo red and calcofluor plates. Colony morphology was inspected at daylight (Congo red) and after exposure to UV light (calcofluor). The color and surface of colonies on Congo red plates indicated the presence of curli or/and cellulose. On calcofluor plates, fluorescent colonies indicated binding of calcofluor to cellulose (paper II).

Detection of bacterial sensitivity to LL-37

To test the sensitivity of clinical *E. coli* strains to the synthetic peptide LL-37 (paper I), a modification of the inhibition zone assay was used. Briefly, wells of 3-mm diameter were punched into the layer of LB broth/agarose with embedded bacteria, and loaded with a sample. After an over night incubation, the diameter of the inhibition zone was measured. In addition, single strain regression analysis was used to express the sensitivity also in clinically used terms of minimal inhibitory concentration (MIC). The relationship between the peptide concentration and diameter of the inhibition zone was tested in series of experiments. The correlation between $\log_{10}$ concentration and the diameter of the zone appeared linear. By regression analysis, we found a correlation between the inhibition zone diameter and MIC, which allowed us to express the sensitivity as MIC.

The MIC and 50% effective bactericidal concentrations (EC_{50}) were also determined using *E. coli* CFTO73 and the synthetic peptides LL-37 and CRAMP (paper I) by broth dilution method in microtiter plates. Briefly, bacteria were cultured

overnight on CLED agar plates and suspended in Mueller-Hinton broth. Bacterial suspension was grown in the presence of different concentrations of the peptides at 37°C for 30 min and for 16 hours. EC_{50} after 30 min was analyzed by plating serial dilutions of the bacteria-peptide solutions on blood agar plates. The MIC was determined after 16 hours as the lowest concentration yielding no visible bacterial growth.

The minimal inhibitory concentration of LL-37 and bacteria producing different extracellular components of biofilm was also determined by broth dilution method (paper II). In order to evaluate the impact of biofilm on bacterial sensitivity to LL-37, bacteria were prepared for the experiment by two different ways, in a planktonic phase and in a phase of broken biofilm as described above. In addition to visual determination of MIC, the viability of bacteria was measured colorimetrically by reduction of Alamar blue (BioSource).

In vitro infection

We infected 1 mm thick kidney pieces with approximate weight of 100 mg (Paper I) and epithelial cell lines when they formed a confluent layer (all papers). The experiments were performed in serum-free medium supplemented with gentamycin in 24-well plates. We added 10^8 bacteria into each well and the plates were incubated at 37 °C, 5% CO_2, 95% O_2 and 80% humidity. After preset time points, we analyzed cell medium for peptides/proteins, and cells for respective mRNA transcripts. Kidney pieces were processed for immunostaining.

Studies on the adhesion, invasion, and persistence of bacteria and on the antimicrobial activity of cells (paper II)

Cells were grown until confluence in the absence of antibiotics in 96-well plates and infected with *E. coli* in serum and antibiotic-free medium. In order to analyze the adherence of bacteria, the cells were at preset time points washed with PBS to remove non-adherent bacteria. Then, we collected bacteria attached to the cells by ice-cold water with Triton X-100. After serial dilution in PBS, lysates were plated on blood agar plates, incubated over-night and bacterial numbers were counted.

To evaluate the ability of epithelial cells to kill bacteria attached at 10 min, all the cells were washed after 10 min of infection to remove non-adherent bacteria. This imitated the situation *in vivo*, where non-attached bacteria are flushed by urine. After

washing non-attached bacteria, the survived bacteria were at preset time points collected by water with Triton X-100.

In order to analyze the ability of bacteria to invade cells and to persist intracellularly, we used gentamycin protection assay with modifications. After 10 min of bacterial infection, all the cells were washed with PBS to remove non-adherent bacteria and the medium was replaced with the new medium containing gentamycin (100 μg/ml). After 24 h, the cells were again washed with PBS and intracellular bacteria collected with water and Triton X-100.

Studies on the bacterial growth in blood and LB broth

Bacterial growth in fresh blood, Lancefield assay, was used to assess the effect of TIMP-1 on the bactericidal activity of blood (Lancefield 1957) (paper IV). Freshly drawn human blood in citrate was mixed with *E. coli* suspension together with different concentrations of TIMP-1. Under similar conditions, an effect of TIMP-1 on the growth of bacteria was evaluated using a common growth substrate, LB broth. In both cases, the mixtures were slowly rotated at 37˚C for 3 hr, and the number of bacteria was analyzed by plating serial dilutions of samples on blood agar plates.

Studies on the functions of granulocytes

In paper IV, we studied the effect of recombinant human TIMP-1 on different functions of normal human granulocytes, namely respiratory burst, spontaneous apoptosis and migration through artificial base membranes.

To analyze the respiratory burst, the cells were first incubated with dichlorofluorescein diacetate (DCFH). DCFH enters the cells where it is trapped after deacetylation by intracellular esterases. After cell activation, DCFH is oxidized by hydrogen peroxide to highly fluorescent 2',7'-dichlorofluorescein. The activation was then stopped by adding the ice-cold PBS and the cells were then analyzed by flow cytometry. Median fluorescence intensity of a cell was considered as a measure of hydrogen peroxide production and thus respiratory burst of granulocytes. The fluorescence increase (percentage) of samples stimulated with TIMP-1 versus non-stimulated (negative-control) samples was calculated.

Granulocytes in the tissue and *in vitro* undergo spontaneous apoptosis. We studied the effect of different concentrations of TIMP-1 on this process. After 24 hours of *in vitro* culture, cells were analyzed for morphology, phosphatidylserine exposure

and caspase-3 activity. Slides for morphological analysis were prepared by cytocentrifugation and stained with May-Grünwald-Giemsa staining and the morphology was analyzed by light microscopy. To measure the phosphatidylserine exposure on the outer leaflet of the cell membrane, we stained the cells by the Annexin V (AV) and cell necrosis was detected by the propidium iodide (PI) method. Double staining with AV and PI gives the opportunity to distinguish early apoptotic (AV+/PI–), late apoptotic/necrotic (AV+/PI+), and necrotic cells (AV–/PI+). The cells could therefore be divided into 4 populations according to AV and PI positivity and negativity and the proportion out of the entire cell population was calculated. Caspase-3 activity in granulocytes was measured by the cell permeable substrate PhiPhiLux (OncoImmunin) and also analyzed by flow cytometry. Median fluorescence intensity was considered as a measure of caspase-3 activity in a cell and a decrease versus control (percentage) was calculated.

The migration of granulocytes was investigated using cell culture inserts with membrane pore size of 3 μm, coated with type IV collagen or with a solubilized basement membrane preparation Matrigel (BD Biosciences). To induce the migration of granulocytes, medium from infected human renal epithelial cells was added to the lower chamber. Granulocytes with different concentrations of TIMP-1 were added to the upper chamber. The transmigrated cells were harvested from the lower chamber at preset time points by EDTA in PBS and the number of transmigrated granulocytes was measured by flow cytometry.

Detection of proteins/peptides

We analyzed urine samples as well as medium from *in vitro* experiments for the presence of proteins/peptides; LL-37/hCAP-18, MPO (paper I), IL-8 (paper II), MMP-9, TIMP-1 (paper III and IV) by the ELISA, and LL-37/hCAP-18 also by western blot analysis (paper I). Healthy renal tissue from human kidneys, as well as kidneys and urinary bladders from healthy mice and mice during experimental pyelonephritis were fixed in paraformaldehyde, embedded in paraffin, sectioned, stained using different antibodies and visualized either colorimetrically (paper I and III) or by immunofluorescent amplification (paper I).

Detection of mRNA transcripts

In order to detect mRNA transcripts in the cells and tissues, we isolated total RNA using Trizol (Invitrogen) from healthy samples and cells as well as from samples at

preset time points of *in vitro* infection. After reverse transcription, cDNA was amplified by PCR. PCR products were separated by electrophoresis in agarose gels, stained by ethidium bromide, and visualized under UV light (paper III). Transcript for LL-37/hCAP-18 was analyzed by real-time PCR and quantified in correlation to the amount of total RNA in a sample or to the expression of the house-keeping gene *GAPDH* (paper I).

Data analysis

Each set of experiments using normal human cells or tissue samples was repeated with cells or samples from at least 3 patients. Each experiment was performed at least three times. For the data with normal distribution, t-test for independent samples or analysis of variance (ANOVA) was used. Mann-Whitney U test, Kruskal-Wallis ANOVA or Spearman's rank correlation test were employed for the rest of the data. Differences with p level less than 0.05 were considered as statistically significant.

RESULTS

The antimicrobial peptide cathelicidin in the urinary tract (paper I)

In paper I, we describe the production and role of the cathelicidin antimicrobial peptide in epithelial cells of the urinary tract. We found low levels of cathelicidin LL-37/hCAP-18 in urine of healthy children and increased levels in children having acute UTI. Low correlation of urinary cathelicidin levels with the number of leukocytes and the leukocyte marker myeloperoxidase indicated additional non-leukocyte origin of cathelicidin in urine. In search for local sources of the peptide, we examined pieces of healthy renal tissue from nephrectomized patients but we found only week indication of low constitutive production of LL-37/hCAP-18. When we incubated renal pieces with uropathogenic *E. coli*, cathelicidin became clearly visible in the cytoplasm of renal tubular cells. These findings were strengthened by the *in vitro* experiments. Both uroepithelial and renal epithelial cells already within 5 minutes increased the levels of cathelicidin mRNA transcript and released cathelicidin peptide into the medium. In mouse kidneys, we detected production of mouse cathelicidin CRAMP during experimental UTI. Similarly as in humans, CRAMP had two sources, epithelial cells and neutrophils. Mice could therefore be used as a model of human disease.

To investigate the relevance of cathelicidin production for the protection of the urinary tract, we challenged mice deficient in cathelicidin with uropathogenic *E. coli*. Cathelicidin producing, normal mice were more difficult to infect as compared with cathelicidin deficient animals. The protection against infection was mediated by epithelium and not neutrophil-derived cathelicidin, which we tested by two different approaches. First, we studied antimicrobial activity of the urinary bladder mucosa already after one hour of infection, which is too short time for the migration of neutrophils. We found significantly less bacteria attached to the mucosa of cathelicidin positive bladders as compared with the deficient ones. This finding was confirmed by *in vitro* experiments where renal epithelial cells from cathelicidin-producing mice killed attaching bacteria more than did cells from cathelicidin-deficient animals. As a second approach to specifically test the relevance of epithelial cathelicidin, we induced temporary neutropenia in animals by treatment with monoclonal neutrophil-specific antibody. Neutropenic animals were still more

difficult to infect if they produced cathelicidin, confirming the role of cathelicidin produced by epithelial cells.

In addition to the higher infection rate, once cathelicidin-deficient animals got infection, they suffered from more severe disease, their kidneys were more swollen and contained more bacteria than kidneys from cathelicidin-producing animals. These effects seemed to be influenced by the cathelicidin originated from neutrophils since the differences were observed only in the presence of neutrophils.

We also evaluated the relevance of the cathelicidin from the perspective of the pathogen, uropathogenic *E. coli*. We isolated *E. coli* strains from children with UTI and tested them for the sensitivity to the synthetic cathelicidin peptide LL-37. Clinical *E. coli* strains that were more resistant to LL-37 caused more invasive urinary tract infections than did susceptible strains. Thus, cathelicidin appears to be a key factor of mucosal immunity in the urinary tract.

Biofilm production by uropathogenic *E. coli* (paper II)

In the second study, we analyzed uropathogenic *E. coli* strains from non-complicated community acquired UTI for biofilm formation and compared them with commensal strains isolated from gastrointestinal tract of healthy individuals. The majority of uropathogenic *E. coli* produced biofilm and quantitatively more than commensal strains. However, the composition of extracellular matrix components of biofilms from both uropathogenic and commensal bacteria was very similar, consisting of curli fimbriae and cellulose.

We chose a typical uropathogenic *E. coli* strain producing both curli and cellulose and prepared its isogenic mutants deficient in curli, cellulose and both. We infected uroepithelial and renal epithelial cells *in vitro* with wild type bacteria and their mutants and studied the role of curli or/and cellulose on pathogen-host interactions. Curli fimbriae promoted adherence of bacteria to uroepithelial and renal epithelial cells. Notably, the increased adherence was not accompanied by increased induction of IL-8 by the cells, and similar result was found also *in vivo* where the production of biofilm correlated with lower levels of IL-8 in urine. The presence of cellulose, on the other hand, decreased both adherence to cells and IL-8 induction. In addition to increased adherence, 'curliated' *E. coli* better survived in the antimicrobial environment on the surface of epithelial cells as well as intracellularly. Since in the previous project (paper

I) we described the important role of cathelicidin for the mucosal immunity of the urinary tract, we wanted to know if the found increased bacterial survival could be explained by the resistance to cathelicidin. And indeed, when bacteria produced both curli and cellulose, they became more resistant to cathelicidin LL-37.

Interestingly, when we incubated bacteria with LL-37 in concentrations lower than minimal inhibitory concentration, the formation of biofilm was significantly inhibited.

MMP-9 and TIMP-1 during acute pyelonephritis and renal scarring (Paper III)

In the third paper, we demonstrated a significant increase of MMP-9 and TIMP-1 in the urine of children with acute pyelonephritis. Using an animal model of ascendant pyelonephritis and cell culture experiments, we studied the cellular localization and kinetics of MMP-9 and TIMP-1 production during infection. In mice, both proteins were produced mainly by leukocytes and TIMP-1 also by resident kidney cells. In accordance with the animal model, human monocytes produced *in vitro* both MMP-9 and TIMP-1 and resident kidney cells only TIMP-1. After bacterial infection, MMP-9 was induced and TIMP-1 suppressed in monocytes, resulting in net increase of MMP-9/TIMP-1 ratio. The constitutive production of TIMP-1 in epithelial cells was, in contrast, maintained *in vitro* and stimulated *in vivo*. These data suggest that MMP-9 and TIMP-1 are produced by different cells, both resident renal cells and professional immune cells, during acute pyelonephritis, and may have various functions depending on the site of production.

Out of 40 children with pyelonephritis, 23 had higher urinary TIMP-1 than MMP-9 levels. These children had significantly more severe changes in both acute and follow-up scintigraphy scans indicating a higher degree of acute tissue damage and renal scarring. Thus, our findings suggest an association between TIMP-1 and the process of renal scarring.

Effects of TIMP-1 during acute inflammation (Paper IV)

We sought to elucidate potential profibrotic mechanisms of TIMP-1 during the acute phase of pyelonephritis.

First, we studied the growth of *E. coli* in LB broth and in fresh human blood with different concentrations of recombinant TIMP-1, to distinguish two possible effects of TIMP-1, effect on bacteria and effect on the immune defense to them. TIMP-1 did not influence the growth of bacteria in LB broth but increased the antimicrobial activity of fresh human blood. To deeper analyze the observed effect of TIMP-1 on the immune system, we isolated granulocytes, the majority of which were neutrophils, from healthy volunteers and studied their functions during acute inflammation in the presence of recombinant TIMP-1. In order to mimic the situation *in vivo*, we infected renal epithelial cells with *E. coli* and the medium from infected cells was then used to culture granulocytes. TIMP-1 stimulated granulocytes and increased their respiratory burst. TIMP-1 also influenced several features of spontaneous apoptosis of granulocytes, morphological changes, phosphatidylserin exposure and caspase-3 activity, which resulted in their prolonged life-span. In addition, TIMP-1 inhibited the migration of granulocytes through artificial base membranes *in vitro*. Clinical data from children with pyelonephritis strengthened the *in vitro* findings since the urinary MMP-9/TIMP-1 ratios correlated with the degree of leukocyte transmigration during pyelonephritis. Putting all the findings about TIMP-1 together, TIMP-1 activates granulocytes and prolongs their stay in the tissue by blocking of both their apoptosis and migration to the urine. The result is an ‘entrapment’ of activated granulocytes.

DISCUSSION

Urinary tract infection is a dynamic event, which involves complex interactions of the microbial pathogen with the human host. In this thesis, we present four papers, where we studied different aspects of UTI; the mechanism, which helps to protect the urinary tract against bacterial infection (paper I); the tactic of the pathogen to colonize the urinary tract and to evade and resist the defense mechanisms (paper II); and once the UTI occurs, the determinants of the proper healing or scarring in the tissue after infection (papers III and IV).

In paper I, our focus was on the mucosal immunity in the urinary tract. It is striking how well the urinary tract resists microbial growth considering that it empties close to the rectum. Moreover, bacteria are frequently found in urine (Hooton et al. 2000b). Still, it seems to be very difficult to establish urinary tract infection. After administering bacteria into the urinary tract of healthy volunteers, microbes are rapidly eliminated (Cox et al. 1961) and very high bacterial concentrations are needed to induce experimental UTI in animals (Hagberg et al. 1983).

Urine flow together with anatomical properties of the urinary tract was traditionally considered to be the most important defense mechanism, which keeps this niche sterile except for the external part of the urethra. However, mathematical simulation and in vivo models (Cox & Hinman 1961; Gordon et al. 1992) suggested that emptying of the bladder is only one of the defense tools, and additional antibacterial factors are essential (Roberts 1996). In line with these experiments, the mucosa of the urinary bladder has been shown to possess antimicrobial properties *in vitro* (Norden et al. 1968). Later on, a number of molecules inhibiting the growth of bacteria in the urinary tract have been identified. The Tamm-Horsfall protein (Bates et al. 2004; Serafini-Cessi et al. 2003), secretory IgA (Bueler et al. 1995; Fliedner et al. 1986) and low molecular weight sugars (Mulvey et al. 2000) can block the adherence of uropathogens. Lactoferrin (Abrink et al. 2000) inhibits the growth of bacteria by decreasing the accessibility of iron. Both main groups of antimicrobial peptides in mammals, defensins and cathelicidins were found in kidneys (Bals et al. 1998; Valore et al. 1998), and the antimicrobial peptide β-defensin-1 has been suggested to be the main antimicrobial peptide of urogenital tissues (Valore et al. 1998). In our study, we

investigated the role of the antimicrobial peptide cathelicidin, which was shown to be effective against the most common uropathogens (Turner et al. 1998).

We found that low levels of cathelicidin are produced constitutively in epithelial cells of the urinary tract and released into urine. The function of cathelicidin in health does not therefore seem to be to sterilize the urine. This may have several reasons. First, high levels of cathelicidin are cytotoxic for eukaryotic cells (Johansson et al. 1998). In addition, not all bacteria are harmful for the human body even if present in the urinary tract. An interesting example is asymptomatic bacteriuria, which could be even beneficial for the host as non-pathogenic bacteria out compete uropathogens (Roos et al. 2006). It has also been hypothesized that levels of cathelicidin limiting the growth of bacteria in the urine might promote the development of bacterial resistance to the peptide (Perron et al. 2006). Notably, as soon as bacteria attach to the cells, cathelicidin is produced and released within minutes. Attached bacteria can therefore be attacked by the mature peptide already before they can divide. Such a fast response has not been observed before and indicates multilevel regulation of the transcription, translation and release of the peptide. The fast increase of antimicrobial activity only after bacteria attach to the cells, the 'compartmentalization' of the mucosal defense may resemble the situation in the gut, which can carry numbers of bacteria without attacking them. However, as soon as bacteria reach a thin antimicrobial layer that covers the mucosa, they are rapidly eliminated (Iimura et al. 2005).

We identified two main sources of cathelicidin during infection, epithelial cells and neutrophils. Epithelial cathelicidin seems to be the first line of defense. Cathelicidin deficient animals were easier to infect, irrespectively of the presence of neutrophils. Epithelial defense is in many cases enough to stop infection. Still, bacteria sometimes win the 'first battle' of epithelial defense. Then, the epithelium needs to recruit the help of professional immune cells. In this case, cathelicidin is still important, first as a chemokine to attract and activate immune cells (Kurosaka et al. 2005; Tjabringa et al. 2006) or as an effector of neutrophil-mediated killing (Sorensen et al. 1997). Cathelicidin thus appears to be a key factor of several parts of the innate immunity of the urinary tract. It remains to be elucidated whether there are differences in the levels of cathelicidin between individuals and if these differences could explain the predisposition of some people to UTI and its recurrences.

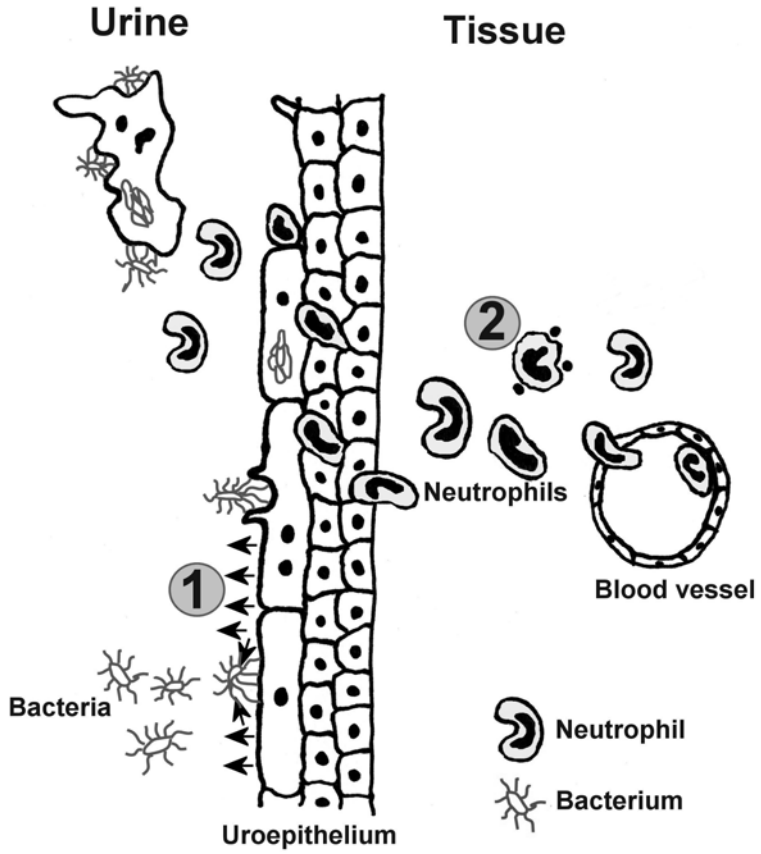

Figure 3. *Cathelicidin in the protection of the urinary tract.* (1) Cathelicidin produced by epithelial cells serves as a first line of defense against attaching bacteria. (2) Cathelicidin in the granules of neutrophils comes as a second wave of defense.

The production of antimicrobial peptides has been reported in virtually all groups of organisms, including bacteria, fungi, plants and animals (Zasloff 2002). Antimicrobial peptides are subjects of positive selection and they are one of the most rapidly evolving groups of mammalian proteins, with major differences between species (Peschel et al. 2006). Antimicrobial peptides have for millions of years been evolving together with the respective pathogens. Still, they remain effective throughout the course of evolution. In contrast, *in vitro* exposure of bacteria to slowly increasing concentration of antimicrobial peptides resulted in the development of resistance to the peptide (Perron et al. 2006). Understanding the processes that have resulted in antimicrobial peptides remaining effective *in vivo* is of particular importance in the era of emergence of antibiotic resistance (Levy et al. 2004) and need for new effective antimicrobials.

In the second study, we sought to investigate the biofilm formation by *E. coli* causing community-acquired UTI. It has been recognized that bacteria in their natural milieu seldom grow as non-differentiated, single cell organisms. Instead, they form multicellular communities, biofilms, which show coordinated behavior (Shapiro 1998). Biofilms may offer strategic advantages in comparison to single cells such as increased resistance to mechanical forces, lack of nutrients, different physicochemical properties of the environment, attacks of the immune system and exogenous antibiotics (Hall-Stoodley et al. 2004; Suthereland et al. 2004). Classically, formation of biofilm includes adherence of a microorganism to a surface or to each other, a change in gene expression resulting in a different phenotype from the planktonic state; and formation of an extracellular matrix (Costerton et al. 1995). This scenario has been described for biofilms growing on abiotic surfaces, e.g. in UTI in the presence of indwelling catheters (Trautner & Darouiche 2004). However, bacteria appear to form multicellular communities also on epithelial surfaces (Costerton et al. 1999; Probert et al. 2002) as well as intracellularly (Anderson et al. 2003), which seems to be important especially for persistent and recurrent infections.

We found that the majority of *E. coli* strains isolated from non-complicated UTI produced biofilm *in vitro* and quantitatively more than commensal bacteria. The main extracellular matrix components of both uropathogenic and commensal *E. coli* strains were the same, curli fimbriae and cellulose. Curli fimbriae are the proteinaceous component of the extracellular matrix of bacteria from the family Enterobacteriacae (Olsen et al. 1989). Cellulose, the most abundant organic polymer found in nature is produced mainly by plants. Most knowledge about the biological role of bacterial cellulose has been gathered about soil bacteria of the family Rhizobiacae, where cellulose is required for the firm adherence plus aggregation of bacteria at the root hair tip of plants (Romling 2002). Although the production of cellulose is common also among other bacterial species its biological function in these organisms is still not clear. When cellulose is expressed together with curli fimbriae, the two substances form a highly inert, hydrophobic extracellular matrix around the bacteria (Romling 2005; Zogaj et al. 2001).

In our study, expression of curli increased adherence of bacteria to uroepithelial and renal epithelial cells. This adherence was not accompanied by increased stimulation of the cells as measured by IL-8 release. Thus, the adherence by curli seems to differ from adherence by type-1 and P-fimbriae, which both induce massive inflammatory

reaction of the epithelium (Sauer et al. 2000). Interestingly, some bacterial strains are able to persist successfully within the urinary tract without induction of inflammatory response (Klemm et al. 2006; Roos et al. 2006) but so far the mechanisms of such bacterial colonization have not been explained. Co-expression of curli with another ECM component, cellulose, even decreased the IL-8 response. Bacterial cellulose displays special physicochemical characteristics and when used as treatment of wounds it does not induce inflammation (Czaja et al. 2006). The precise mechanisms of the effects of bacterial cellulose remain to be elucidated. One can speculate that 'biologically inert' cellulose covers antigen determinants of bacteria and thus protects them from immune recognition.

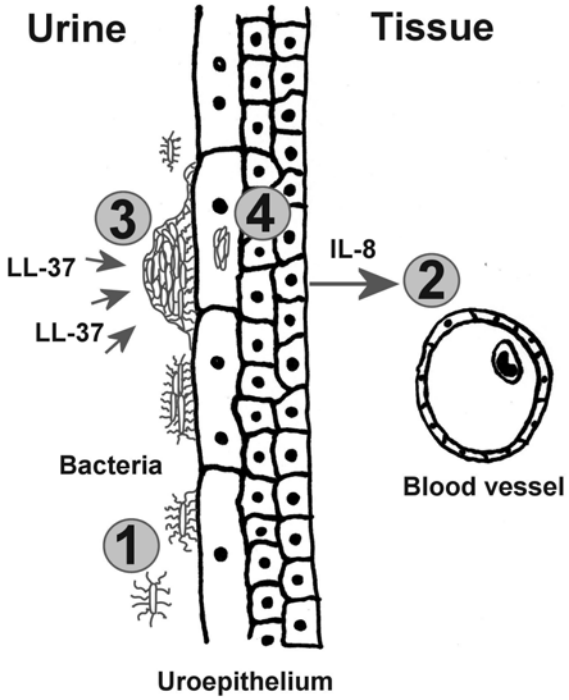

Figure 4. *Effects of E. coli biofilm.* (1) Increased adherence, (2) Decreased induction of IL-8, (3) Resistance to cathelicidin LL-37, (4) Invasion of cells and persistence intracellularly.

In addition to immune evasion, production of biofilm increased the resistance to the antimicrobial peptide cathelicidin and the survival of bacteria in the antimicrobial environment in close proximity to epithelial cells and intracellularly. All the described effects of the *E. coli* biofilms seem to promote the adherence and persistence of bacteria in the urinary tract. Biofilm production might therefore be a virulence factor predisposing to persistent and recurrent UTIs.

Interestingly, concentrations of cathelicidin lower than MIC inhibited the biofilm formation by *E. coli*. This is in line with a previous finding about another

component on the innate immune defense of epithelium, lactoferrin (Singh et al. 2002). Exogenous antibiotics, in contrast, seem to stimulate biofilm formation (Hoffman et al. 2005). Thus, our study not only suggests how bacteria, by forming biofilm, might evade the immune system and cause persistent or recurrent infections. We also suggest how the innate immune system, namely antimicrobial peptide cathelicidin, protects the urinary tract by inhibiting the biofilm formation by uropathogenic *E. coli*. This result opens new possibilities for the treatment and prevention of recurrent urinary tract infections, for example by increasing the levels of antimicrobial peptides (Liu et al. 2006; Raqib et al. 2006; Zasloff 2006a; Zasloff 2006b).

In papers III and IV, renal scarring was in focus. More than one third of patients with pyelonephritis develop renal scars, and scaring may be connected with severe consequences (Martinell et al. 1990; van der Heijden et al. 2004; Wennerstrom et al. 2000a; Wennerstrom et al. 2000b). Still, pathogenesis of the scarring process in the kidney is not completely understood. In paper III, we investigated the role of MMP-9 and TIMP-1. MMP-9 is active against collagen IV, abundant in parenchymal organs like kidney and TIMP-1 is the main inhibitor of MMP-9.

Renal scarring after UTI is triggered by the presence of bacteria in the kidney. Epithelial cells are the first cells in contact with bacteria. If the epithelial defenses are not able to eliminate the bacteria, epithelium produces chemokines and cytokines to recruit 'professional help'. As a result, professional immune cells, in the first hand neutrophils, migrate and accumulate in the site of infection (Haraoka et al. 1999). MMP-9 and TIMP-1 are known to be produced by immune system cells to facilitate their migration (Opdenakker et al. 2001). At the site of infection neutrophils phagocyte bacteria and release the toxic content of their granules. The side effect of such reaction is local destruction of the tissue (Bille & Glauser 1982). Cytokines also cause multiplication and activation of renal interstitial fibroblasts as well as dedifferentiation of epithelial cells by a process called epithelial-mesenchymal transition or transformation (Iwano et al. 2002). The cell activation and proliferation is considered to be an initiating event in renal fibrosis, and has similar features irrespectively of the triggering agent. This induction phase is followed by an increased matrix production and reduced matrix degradation, which results in a deposition of extracellular matrix components, mainly collagen (Muller et al. 2000).

After the infectious agent is eliminated, either by the immune system or by exogenous antibiotics, the destructed tissue with accumulated matrix is digested and removed during the phase of healing (Eddy 2000; Eikmans et al. 2003). MMP-9/TIMP-1 may also participate in the healing process by digestion of the collagen. It is generally accepted that acute defects in the renal parenchyma could be completely resolved within a period of 6 months. If the defects persist longer, they are considered to be scars (Ditchfield et al. 2002).

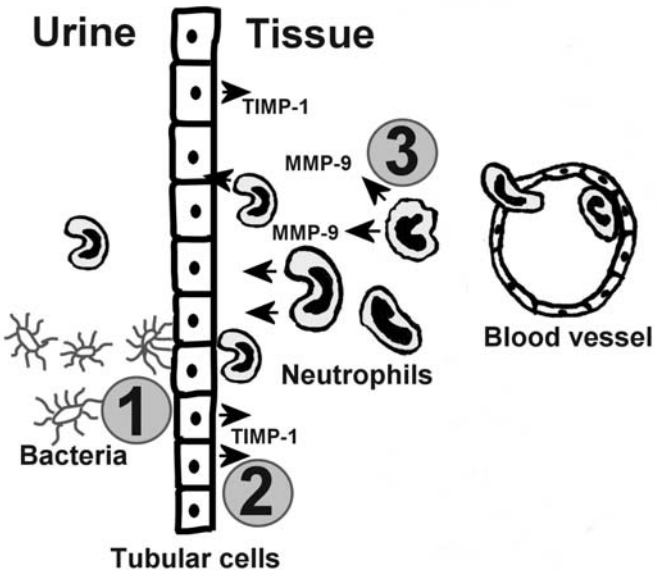

Figure 5. *MMP-9 and TIMP-1 in acute pyelonephritis.* (1) After contact with bacteria, (2) tubular cells in the kidney generate TIMP-1. (3) Professional immune cells, e.g. neutrophil granulocytes, produce MMP-9 in. Neutrophils thus digest the tissue and migrate to urine to kill bacteria. The ratio between MMP-9 and TIMP-1 determines the degree of transmigration of neutrophils and the degree of tissue damage.

There could be two scenarios in the pathogenesis of renal scarring. One possibility is that the tissue is during acute inflammation destroyed excessively and normal healing mechanisms are not strong enough to replace the damage. For example, after direct inoculation of *E. coli* into the kidneys of rats, contraction and collapse of the tubulointerstitial parenchyma had a greater influence than new collagen production on final fibrosis (Hewitson et al. 1998). Such mechanism has been suggested also in post infective renal scarring caused by defective transmigration of granulocytes (Hang et al. 2000). In line with this, it has previously been shown that mouse kidneys after experimental acute pyelonephritis were smaller than those from uninfected animals (Khalil et al. 2000a). Likewise, it seems that the end-stage kidney is rather shrunken, not enlarged or full of collagen (Heptinstall 1992).

The second possibility is that scarring could be a result of prolonged activation and proliferation of different cell types, mainly interstitial fibroblasts, leading to a permanent deposition of ECM components. Decreased ability to degrade accumulated matrix could lead to the same outcome (Gonzalez-Avila et al. 1998). This mechanism has been proposed in the pathogenesis of renal fibrosis due to many non-infectious cases of renal fibrosis, e.g. glomerulonephritis or diabetic nephropathy (Eddy 2001; Mo et al. 1999).

In both possible scenarios, MMP-9 and TIMP-1 may be involved. The possible actions are increased digestion of the tissue during acute inflammation or decreased healing by decreased digestion of the collagen. Moreover, MMP-9 and TIMP-1 may influence the intensity and duration of inflammation by processing and degradation of various cytokines and growth factors (Bergers et al. 2000; Schonbeck et al. 1998).

Both MMP-9 and TIMP-1 were induced during acute UTI in immune system cells and in resident renal cells. In immune system cells, infection was accompanied with increased MMP-9/TIMP-1 ratio, which promotes their migration (Opdenakker et al. 2001). In resident renal cells, on the contrary, TIMP-1 was induced. Similar reaction was observed during infection in the eye and was attributed to the protection against proteolytic activity during inflammation (Kernacki et al. 1999). Thus, it seems that the balance between MMP-9 and TIMP-1 may determine the intensity of inflammation and the destruction of the tissue. Urinary levels of TIMP-1 higher than MMP-9 correlated with more severe acute renal damage and the development of renal scarring. This result was surprising because the opposite, increased MMP-9/TIMP-1 ratio was previously associated with acute damage during infections (Hewitson et al. 1998; Paemen et al. 1997). On the other hand, our data are in line with studies showing that decreased MMP-9/TIMP-1 ratio is connected with prolonged decreased proteolytic activity, accumulation of collagen, and fibrosis in different organs (Eddy 2001; Hayashi et al. 1996; Iredale et al. 1998). The latter mechanism could not, however, explain our findings, since accumulation of collagen takes place later, not during the acute phase of inflammation (Hewitson et al. 1998).

Therefore, in paper IV we studied other possible effects how TIMP-1 could affect renal scarring already during the acute phase of inflammation. We found that TIMP-1 prolongs the stay of granulocytes in the tissue by blocking of both granulocyte apoptosis and migration to urine. Prolonged life of granulocytes in the tissue was previously connected with more severe inflammation (Hofman 2004) and tissue injury.

Similarly, mutation or absence of CXCR1 receptor, needed for granulocyte transmigration through epithelial cells, led to accumulation of granulocytes in the tissue and renal scarring (Hang et al. 2000). In addition to 'entrapment' of granulocytes, TIMP-1 activates them. Too high levels of TIMP-1 might therefore lead to excessive destruction of renal parenchyma and consequent renal scarring. These series of events can explain the profibrotic effect of TIMP-1 already during acute inflammation.

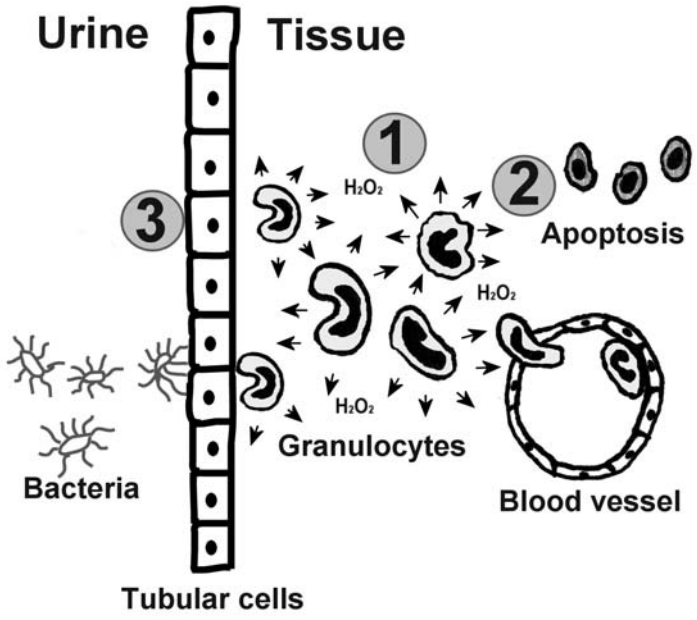

Figure 6. *Effects of TIMP-1 during acute inflammation.* TIMP-1 (1) activates respiratory burst of neutrophil granulocytes, (2) protects granulocytes from spontaneous apoptosis, and (3) inhibits migration of granulocytes into urine.

Certain degree of tissue destruction seems to be inevitable side effect of our immune defense. The protease/antiprotease imbalance may be involved in pathogenesis of many diseases. Therefore, antiproteolytic treatment has been suggested in order to protect different tissues. However, our data and data from others suggest that some caution should be warranted. For example, in different disease, cerebral stroke, antiprotease treatment led to increased tissue damage and impaired functional recovery (Zhao et al. 2006). As results of paper III and IV, as well as by others show, the situation during inflammation appears to be more complex. Specific proteases and protease inhibitors might have multiple functions (Chirco et al. 2006) and their role may differ in different stages of a disease. TIMP-1 is a good example. It apparently protects the tissues from proteolysis. On the other hand, too high concentrations of TIMP-1 might also increase the tissue damage during acute bacterial infection by activation and 'entrapment' of granulocytes in the tissue.

The common theme of all the studies presented in this thesis is an interaction between bacteria and human host during urinary tract infection. The urinary tract is protected against bacteria by the production of cathelicidin, an endogenous antibiotic, which is produced with lightening speed after bacterial attachment (paper I). Bacteria, however, developed mechanisms how to resist this defense. Production of biofilm helps bacteria adhere to cells, not to stimulate the production of IL-8 and be protected against cathelicidin (paper II). Cathelicidin, though, in its low concentrations, inhibits the formation of bacterial biofilm, emphasizing its importance in the protection of the urinary tract (paper II). Bacteria sometimes win the first battle against mucosal immunity. Then, professional help of immune cells is called and more intense inflammation with some inevitable tissue damage is needed. A balance between MMP-9 and TIMP-1 produced during acute inflammation is important for proper healing. If too much of TIMP-1 is produced, the inflammation may lead to renal scarring (paper III). The mechanism of profibrotic action of TIMP-1 seems to be complex, resulting in 'entrapment' and activation of granulocytes with excessive tissue destruction (paper IV).

Data presented in this thesis describe several mechanisms in the pathogenesis of urinary tract infection and postinfective renal scarring. Basal levels of cathelicidin and MMP-9/TIMP-1 may be influenced by environmental factors as well as by genetic variability (Liu et al. 2006; Raqib et al. 2006; Timms et al. 2002; Ye 2000). Further studies are needed to evaluate if the variability in cathelicidin and MMP-9/TIMP-1 may explain the interindividual differences in the susceptibility to urinary infections and renal scarring. Our results also open new possibilities for the treatment of UTI in order to protect the urinary tract from bacteria and the kidney from damage.

CONCLUSIONS

I. Epithelial cells of the urinary tract and kidney constitutively produce the antimicrobial peptide cathelicidin in low levels. After contact with bacteria, the peptide production increases within minutes. Cathelicidin plays and important role in protection of the urinary tract against bacterial infection.

II. Uropathogenic *E. coli* produce biofilm, which may help bacteria adhere to the epithelium, evade and resist innate immune responses. Epithelium, on the other hand, produces cathelicidin, which inhibits biofilm formation by uropathogenic *E. coli*.

III. MMP-9 and TIMP-1 are produced during acute pyelonephritis. A delicate balance between the production of MMP-9 and TIMP-1 is important for proper healing. Relatively higher levels of TIMP-1 are associated with the development of renal scarring.

IV. TIMP-1 during acute pyelonephritis activates granulocytes and causes their 'entrapment' in the tissue. Extensive tissue destruction may then lead to renal scarring.

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