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RENAL CELL DEATH IN URINARY TRACT INFECTIONS: ROLE OF *E.COLI* TOXINS

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To

Wenjie, Bilin
And
My parents

Abstract

Febrile urinary tract infections (UTI) are common bacterial illnesses in children. Several follow up studies have shown that 10-15 % of infants with pyelonephritis will develop permanent renal damage, i.e., renal scars, which in turn may lead to chronic diseases such as hypertension and renal insufficiency. During the last decades, as a result of the tremendous improvement of diagnostic and therapeutic approaches, the incidence of severe complications after UTI has dramatically decreased. However, young age seems to be a risk factor for the development of renal scars. Thus, all children with UTI are followed-up for several years, resulting in high costs for the health care system. In a long run, it might be therefore a benefit to identify early those children with high risk to develop renal scars, a pathological process associated with cell death. This study, mainly by studying the interaction between the toxins from uropathogenic *E.coli* strains and renal cells, was aimed to provide information about the mechanisms that regulated cell survival or death in renal proximal tubular cells.

By using a renal proximal tubular cell line (LLC-PK1 cells), we found that soluble toxins from the uropathogenic *E.coli* ARD6 strain (O6) induce apoptotic cell death in a dose- and time-dependent manner. The expression of Fas receptors and the phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2) were significantly upregulated by *E.coli* soluble toxins. Cell death could be completely inhibited by two specific ERK1/2 inhibitors, but not by a broad caspase inhibitor, zVAD-fmk, implicating a caspase-independent pathway via ERK. We also found that lysophosphatidic acid (LPA) could trigger a survival signal through G-proteins and phosphatidylinositol 3-kinase (PI3K). Moreover, this study demonstrated that inducible nitric oxide synthase (iNOS) protein expression was up regulated in renal tubular cells undergoing *E. coli* toxins-mediated death, whereas endothelial nitric oxide synthase (eNOS) was down regulated. When NOS activity is inhibited by the specific inhibitor of NOS or MEK1, cells were rescued from death. Up-regulation of heme oxygenase-1 (HO-1) by nitric oxide (NO) donor, sodium nitroprusside (SNP) or by the specific activator (hemin) inhibited O6-toxin(s)-induced cell death. Furthermore, incubation with a metabolite of HO-1, carbon monoxide, could counteract the effects of *E.coli*-toxins. The protection by carbon monoxide was associated with up-regulation of p21 protein expression.

We also found that 21% of *E.coli* strains isolated from children admitted to our emergency unit with acute UTI show hemolytic activity. All of these strains induced apoptosis in renal tubular cells. In the non-hemolytic strains, only 45% induced apoptosis in cultured cells. It is

therefore likely that hemolysin is one of the main toxins being responsible for the pro-apoptotic activity. The pro-apoptotic cascade activated by hemolysin can be inhibited by HO-1 activation.

We also used an animal model of fetal growth retardation induced by antenatal exposure to excess dexamethasone (DEX). We isolated renal proximal tubular cells (PTCs) from 20-day-old offspring in DEX-exposed-group (DEX-cells) and control-group (CON-cells). After 4 days in culture, cells were exposed to uropathogenic *E.coli* toxins. We found that cell death rate was higher in DEX-cells than that in CON-cells. Cell death exhibited morphological and biochemical features of apoptosis, such as shrinkage, membrane blebbing, nucleus fragmentation and caspase activation. Conversely, the activity of the antioxidant enzyme catalase was significantly increased in renal cortex homogenate from 20-day-old DEX-rats. Furthermore, we observed that cell death rate induced by hydrogen peroxide was not significantly different between DEX- and CON-cells and that the antioxidant vitamin E could not prevent *E.coli*-induced death. Therefore, oxidative stress does not seem to be the crucial factor for *E.coli* toxins-induced cell death.

In conclusion, activation of ERK mediates *E.coli* toxins-induced renal cell death via the iNOS activation. Caspases, although being activated, are not necessary for cell death, and they act after the ERK signaling at which point cells become committed to cell death or can be rescued. Hemolysin is one of secreted toxins that are involved in apoptosis during UTI. Intrinsic and extrinsic factors such as HO-1 and LPA may protect cells against death. There are long-lasting effects of prenatal glucocorticoids that may predispose renal cells to apoptosis after urinary tract infection.

List of Publications

This thesis is based on the following publications that will be referred to their roman numerals:

I **Ming Chen**, Timo Jahnukainen, Wenjie Bao, Elisabetta Daré, Sandra Ceccatelli and Gianni Celsi. Uropathogenic *Escherichia coli* toxins Induce Caspase-Independent Apoptosis in Renal Proximal Tubular Cells via ERK Signaling. American Journal of Nephrology, 23: 140-151, 2003.

II **Ming Chen**, Wenjie Bao, Roman Aizman, Ping Huang, Olle Aspevall, Lars E Gustafsson, Sandra Ceccatelli and Gianni Celsi. Activation of Extracellular Signal-Regulated Kinase Mediates Apoptosis Induced by Uropathogenic E.coli toxins via nitric oxide synthase: protective role of heme oxygenase-1. The Journal of Infection Diseases, 190: 127-135, 2004.

III **Ming Chen**, Wenjie Bao, Sandra Ceccatelli and Gianni Celsi. Prenatal exposure to high level of glucocorticoids increases the susceptibility of renal proximal tubular cells to apoptosis induced by uropathogenic *Escherichia coli* toxins. American Journal of Nephrology, 24:497-502, 2004.

IV **Ming Chen**, Roshan Tofighi, Wenjie Bao, Olle Aspevall, Timo Jahnukainen, Lars E Gustaffason, Sandra Ceccatelli and Gianni Celsi. Heme oxygenase-1 derived-carbon monoxide prevents apoptosis induced by uropathogenic *Escherichia coli* toxins. Manuscript.

Relevant publications not included in this thesis.

Ahlbom E, Gogvadze V, **Chen Ming,** Celsi G, Ceccatelli S. Prenatal exposure to high levels of glucocorticoids increases the susceptibility of cerebellar granule cells to oxidative stress-induced cell death. Proc. Natl. Acad. Sci. U. S. A. 97(26), 4726-14730, 2000.

B Canlon, S Erichsen, E Nemlander, **Ming Chen**, A Hossain, G Celsi, S Ceccatelli. Alterations in the intrauterine environment by glucocorticoids modifies the developmental programme of the auditory system. European Journal of Neuroscience 17 (10): 2035- 41, 2003.

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Y-H Li, **Ming Chen**, A Brauner, C Zheng, J Skov-Jensen, and K Tullus. *Ureaplasma urealyticum* Induces Human Lung epithelial cells and Macrophages Apoptosis. Biol Neonate, 82:166–173, 2002.

T Jahnukainen, **Ming Chen** and G Celsi. Mechanisms of renal damage due to infection. Pediatric Nephrology, in revision.

M Grönroos, **Ming Chen**, T Jahnukainen, A Capitanio, R Aizman, Gianni C. Methotrexate induces cell swelling and necrosis in renal proximal tubular cells, submitted.

Contents

List of abbreviations

CO	Carbon monoxide
CNF1	Cytotoxic necrotizing factor 1
DEX	Dexamethasone
E.coli	*Escherichia coli*
eNOS	Endothelial nitric oxide synthase
ERK	Extra cellular regulated-protein kinase
H_2O_2	Hydrogen peroxide
hly	Hemolysin
HO-1	Heme oxygenase-1
iNOS	Inducible nitric oxide synthase
LPA	Lysophosphatidic acid
LPS	Lipopolysaccharide
O6	*E.coli* ARD6 (serotype O6K13H1)
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
PARP	Poly-ADP (ribose)-polymerase
PD	PD98059
PFT	Pifithrin-α
PI3K	Phosphatidylinositol 3-kinase
PTCs	Proximal tubular cells
siRNA	Small interference RNA
TLR-4	Toll-like receptor 4
TNF	Tumor necrosis factor
U0	U0126
UTI	Urinary tract infection
Wort	Wortmannin

1. INTRODUCTION

1.1 Urinary tract infections in children

Febrile urinary tract infections (UTIs) are common bacterial illnesses in children (1). Several follow up studies have shown that 10-15 % of infants with UTI will develop permanent renal damage, i.e., renal scars, which in turn may lead to generation of pathological conditions and chronic diseases such as hypertension and renal insufficiency. During the last decades, due to the improvement of diagnostic and therapeutic approaches, the incidence of severe complication after UTIs has dramatically decreased in the Western countries. However, development of complications still occurs in 5-10 % of children with renal scars. Thus, all children with UTI are followed-up for several years, resulting in high costs for the health care system. In a long run, it might be therefore a benefit to identify those children with high risk to develop renal scars, a pathological process that might be associated with cell death.

1.2 *Escherichia coli (E.coli)* as the main uropathogen in UTI

E.coli coli accounts for as many as 80% of all UTIs. *E.coli* that cause UTI are not a random sample of the fecal flora (2). Indeed, there are measurable differences in two broad contexts: a) phylogenic segregation between pathogenic and nonpathogenic strains; b) possession of specialized virulence genes that are often horizontally acquired and are usually absent in nonpathogenic strains. A hallmark of pathogenic *E.coli* is their possession of specialized virulence factors, traits that confer pathogenic potentials and are frequent among commensal strains (3, 4).

1.2.1 E.coli virulence factors:

The proposed virulence factors of uropathogenic *E.coli* include an array of adhesions factors including Type 1, P and S fimbriae as well as adhesins of the Dr family and non-fimbrial adhesins. Toxins such as α-hemolysin and cytotoxic necrotizing factor 1 have been shown to be important in extra-intestinal *E.coli* infection. The aerobactin that helps *E.coli* overcome iron limitation and the polysaccharide capsule that exerts resistance to host killing have also been implicated. However, none of the currently known virulence genes or set of genes can clearly define the prototypic uropathogenic *E.coli*.

***Adhesins*:** Adhesins are indispensable for the establishment of an infection in UTI. Adherence factors not only facilitate the colonization of *E.coli* in the urinary tract and promote *E. coli* persistence in the bladder, which may serve as a reservoir for ascending infection in the

urinary tract (3, 4), but also cause invasions and mucosal inflammation. Various adhesins have been identified functioning via specific receptors. Type 1 fimbriae recognize mannose-containing receptors. The *fim* open reading frame encoding type 1 fimbriae exists in almost all *E. coli* isolates from urinary or fecal specimens. Although Type 1 fimbriae has been suggested to aid in the persistence of *E. coli* in the urinary tract in experimental UTI models (5, 6), there has been little direct epidemiologic evidence for an association between Type 1 fimbriae and UTI infection. Type 1 occurs in virtually all *E. coli.* Allelic variation exists in *fimH*, the gene for lectin subunit of the type I fimbriae.

Sokurenko *et al.* (6) have shown that type 1 fimbriae with different FimH alleles vary in their ability to recognize various mannosides and that only those mediating high levels of adhesion via monomannosyl residues are capable of promoting *E. coli* adhesion to uroepithelial cells. Therefore, it seems that certain variants of type 1 fimbriae may contribute more than others to *E. coli* urovirulence (6). The best studied and perhaps the most important type of adhesin is P fimbriae, which recognizes Toll-like receptor 4 (TLR-4) and glycolipids as receptors. A great many studies have confirmed that P fimbriae, measured either by phenotype or genotype, occurs more frequently among *E. coli* strains causing UTI than from fecal isolates. P fimbriae are the most dominant feature in strains causing pyelonephritis. P fimbriae are expressed in about 80 % of the virulent *E.coli* strains causing pyelonephritis. Similarly, about 31% of the *E. coli* isolates from patients with cystitis possess P fimbriae. Using hybridization to a *pap* gene probe it was found that the percentage of positive probes isolated from pyelonephritis, recurring UTI and fecal samples is 82.4%, 59.3%, 34.2%, respectively (2). These data suggest that the prevalence of P fimbriae varies directly with the severity of UTI. A new P fimbriae subtype was recently identified and is frequently found in *E. coli* isolates from UTIs (7). S fimbriae of *E. coli* bind to sialyl galactosides and are implicated in experimental UTI in rats (8). In some studies, UTI isolates are at least two times more likely to carry S fimbriae genes than fecal isolates (2).

***Toxins*:** Toxins are important virulence factors in a variety of *E. coli* mediated diseases. A mechanism used by pyelonephritogenic strains to promote UTIs is by targeting toxins to the extracytoplasmic region via a secretion system (9). Production of toxins by colonizing *E. coli* may cause an inflammatory response, a possible pathway for UTI symptoms.

Alpha hemolysin (HlyA) and cytotoxic necrotizing factor 1 (CNF1) are two toxins that have been associated with uropathogenic *E. coli*. Using hybridization to an *hly* gene probe, it was

found a significant difference in the presence of *hly* between UTI (31% to 50%) and fecal (15%) isolates (2). CNF1 is also found more often in UTI strains than in fecal strains. Caprioli *et al.* (10) examined 91 UTI isolates and determined that 37% produced both CNF1 and hemolysin, while among 114 isolates from normal stools only 1 produced CNF1.

Accumulating data suggest that approximately less than 50 % of the *E.coli* isolated from the urine of patients with pyelonephritis produce the pore-forming toxin, α-hemolysin. Hemolysin damages the cell membrane by making pore on it, facilitating the passage of bacteria through the epithelial barrier (11, 12). Alpha-hemolysin may also have dual effects on renal tubular cells. High doses cause uncontrolled increase in intracellular calcium concentration, cell lysis and death whereas low concentration induces oscillatory change in intracellular calcium concentration leading to increased cytokine production (13). Jantausch et al (14) showed that urinary IL-8 concentration is increased in children with hemolysin producing *E.coli* UTI. The effect of soluble hemolysin is independent on bacterial adherence. Uropathogenic toxins can also subvert host cell signaling to induce cellular response that facilitates bacterial invasion and colonization. Cytotoxic necrotizing factor1 (CNF1) causes a specific deletion of Rho GTPase through hijacking the proteasome machinery (15) to induce cell mobility, which, in turn, may facilitate the spread of the bacterial infection. CNF1 can also promote epithelial cells macropinocytosis (capture of large material in normal cell) and function as professional macrophages (16).

LPS is a component of the outer membrane of gram-negative bacteria cell wall. It is known to activate host response mechanisms, induce nitric oxide and cytokine production in single cell models, isolated tissues and in whole animal experiments (17). The inflammatory response of LPS is mediated via LPS interaction with specific LPS CD14 receptor and toll-like receptor 4 (TLR-4). TLR gene polymorphisms may influence susceptibility to UTI. Experimental knockout animals lacking TLR-4 are highly susceptible to UTI due to hyporesponsiveness to LPS and absence of neutrophil recruitment (18). The expression of TLR-4 may play a crucial role in the LPS induced local immune response, bacterial clearence and cell damage. TLR-4 has been shown to be expressed in the loop of Henle, renal distal tubular cells, and collecting ducts of mice (19), but not in the human proximal tubular cell line (20). More recent data in human kidney biopsies have evidenced TLR4 but not CD14 expression in epithelial cells lining the entire urinary tract and in the renal tubular epithelium (21). In addition to the immunogenic activity, LPS has been shown to have direct toxic effect on renal cells via its biologically active compound lipid A (17) by rapid release of intracellular calcium and

activation of nitric oxide synthase and production of nitric oxide. Antioxidants are able to prevent this LPS mediated cell death in vitro, but no clinical evidence about protective effect of antioxidants against renal scarring has been shown.

Therefore, identification of *E.coli* toxins that cause renal damages in UTI is of clinical relevance and is still under active investigation. Most recently, a new toxin, secreted autotransporter toxin (Sat), was identified as a virulence factor of uropathogenic *E. coli* (22). Sat, a vacuolating cytotoxin, elicits renal cell vacuolization and necrotic cell death in kidney epithelium during upper urinary tract infection (23). Using a *sat* gene probe, Guyer *et al.* found that 38 out of 67 (55%) pyelonephritis isolates but only six out of 27 (22%) fecal isolates carried the *sat* gene (22).

1.3 Effects of *E.coli* infection on renal cells

As previously discussed, the insult of *E.coli* on renal epithelial cells requires bacterial adhesion (through fimbriae) (3, 4) and exposure to bacterial toxin(s), such as LPS, hemolysin, etc. The resulting inflammatory response can be considered as double-edged sword. It is necessary for eradication of pathogenic bacteria but it may also lead to cell damage and permanent scar formation. Development of hypertension has been shown in 10% of children and young adults with pyelonephritic renal scarring. The risk correlates to the extend of damage; 15 to 30% of children with bilateral scaring develop hypertension within 10 years (24-26).

1.3.1 Inflammatory response: Bacterial adherence is followed by mucosal inflammatory response including activation of complement cascade, cytokine production, aggregation of polymorphonuclear cells (PMNs), and capillary obstruction. Neutrophils will reach the tubular lumen and become active phagocytes, opposing the bacteria by means of complement activation by the alterative pathway. Once the polymorphonuclear cells reach the bacteria, phagocytosis begins. It was suggested that inflammatory events are directly responsible for renal damage following infection (27), as the extent of renal damage is directly correlated with the number of neutrophils and can be modulated by treatment with corticosteroids. There are also evidences showing that bacteria associated with renal scarring cause more active release of superoxide and other toxic oxygen radicals from the surface of the phagocyte than within the phagosome (28). Superoxide production may lead to production of other toxic radicals such as hydrogen peroxide, hydroxyl radicals and single oxygen. These reactions will all eradicate bacteria in the phagosome (29), but they can damage the renal tubules as well. These toxic substances are also released into

tubular lumen by macrophages during phagocytosis process in order to kill bacteria. The reactive oxygen radicals have been also shown to stimulate the growth of fibroblasts, thus promoting fibrosis and scar formation. Thus, the free radical of oxygen causes cellular damage by at least two mechanisms: damage of DNA and damage to the cell membrane by lipid peroxidation. The overall effect on the cell membrane is to decrease its fluidity and destabilize membrane receptors, as well as leading to cell death (30).

1.3.2 Renal cell death following UTI: The influence of fimbriae on renal cell damage is not fully clear. Type 1 pili is known to induce apoptosis in mice bladder cells (31). However, this phenomenon is believed to be involved in part of the host defense mechanisms. In addition, P-fimbriae, type I fimbria, and F1C fimbriae trigger direct inflammatory responses by activating epithelial secretion of IL-8. The effect of type 1 fimbriae is lipopolysaccharide (LPS) dependent whereas P fimbriated bacteria act via LPS independent ceramide mediated signaling (32-34).

Granulocytic aggregation together with bacterial toxins leads to endothelium damage, capillary obstruction, and hypoxia and cell death. Hypoperfusion and tissue hypoxia is followed by a reperfusion period, which is characterized by production of free radicals, superoxide, hydrogen peroxide, hydroxyl radical, singlet oxygen, and myeloperoxidase by renal tubular cells. Oxygen radicals cause cell membrane injury leading finally to cell death.

Accumulating evidence indicates that excessive production of NO plays also a pathogenic role in both acute and chronic models of inflammation. During the inflammatory response to infection, toxins induce rapid up-regulation of iNOS (35). A new working hypothesis has been evidenced. The constitutive forms of NOS including neuron NOS (nNOS) and endothelial NOS (eNOS) are critical to normal physiology and inhibition of these enzymes causes damage. On the other side, induction of the inducible nitric oxide synthase (iNOS) is harmful and specific inhibition of this enzyme is beneficial (36). Increased concentration of nitric oxide (NO) in rat renal tissue, bladder and urine has been shown after *E.coli* infection (37, 38). NO may form peroxynitrates with oxygen radicals leading to cell membrane injury and apoptotic cell death. Monocytes/macrophages release tumor necrosis factor (TNF), oxygen radical species and NO, which have been suggested to induce apoptosis in renal epithelial cells exposed to *E.coli* or their toxins (35, 39-41).

1.4 Cell death: apoptosis versus necrosis

The two morphologically distinct forms of cell death recognized are referred to as apoptosis and necrosis (42). Apoptosis, or one programmed cell death, is an active highly regulated process that involves a cascade of intracellular events to eliminate damaged and unwanted cells. It is regulated by several internal and external signals. Apoptosis is characterized morphologically by cell shrinkage, chromatin condensation, DNA fragmentation, plasma membrane and nuclear envelop blebbing and finally formation of membrane-enclosed vesicles containing intracellular materials, referred to as apoptotic bodies (42, 43). The composition of the cell membrane is altered, allowing the rapid recognition and engulfment of apoptotic cells / bodies by healthy adjacent cells or macrophages. The importance of this event is to prevent the spilling of cellular contents and consequent inflammatory response (44). Necrosis, on the contrary, is a passive and uncontrolled process, which accidentally occurs in response to acute injury of the cells. It is characterized morphologically by swelling of the cell and its organelles due to a loss of ion balance and uptake of water, leading to rupture of the plasma membrane and cell lysis (45). The release of the cellular contents into the intercellular milieu will cause further damage to neighboring cells and tissue and evoke an inflammatory response.

1.4.1 Molecular mechanism of apoptosis: There are at least two pathways leading to apoptosis, the receptor-mediated killing and the mitochondria-mediated cell death (46). In the receptor-mediated apoptosis the process is initiated by binding of ligands to "Death Receptors" (DR) located in the cell membrane. Members of this receptor family are FAS/CD95, tumor necrosis factor-α (TNF-α), DR4 and DR5 that bind to TNF-α-related apoptosis inducing ligand (TRAIL). Death receptors are characterized by a conserved cytoplasmic motif (death domain) capable of binding to cytoplasmic adaptor molecules. The recruitment of these molecules results in the activation of caspase-8, an initiator caspase that may directly cleave and activate the effector caspase, e.g. caspase 3. This pathway occurs predominantly in certain cellular models, e.g. type I cells. In other cells (type II cells), caspase 8 cleaves instead the cytoplasmic protein Bid, generating a fragment that activates a mitochondrial pathway. Other enzymes, e.g. lysosomal enzymes, calpains and granzyme B, can also cleave Bid, although the physiological significance of this cleavage is not fully understood. During the past decade, accumulating evidence suggested that lysosomal rupture might be an early event in apoptosis caused by oxidative stress or other inducers (47-53). Lysosome-mediated apoptosis can be induced following minor lysosomal rupture, while necrosis follows upon extensive lysosomal rupture. Lytic pro-enzymes stimulated by

lysosomal enzymes in turn will promote further destabilization of both lysosomes and mitochondria (53, 54).

Signals that activate apoptosis via mitochondria cause the release of mitochondrial factors that may trigger cell death via caspase pathways or by stimulation of caspase-independent degradation events**.** Cytochrome *c*, which is part of the mitochondrial electron transport chain, can be released into the cytoplasm, where it interacts with apoptotic protease activating factor-1 (Apaf-1) via a specific caspase recruitment domaine (CARD). In the presence of dATP/ATP, CARD recruits procaspase 9 to form the apoptosome complex. Within this complex caspase 9 is activated and initiates the activation of effector caspases, such as caspase 3 and 7. Mitochondria can release other factors as well, including Second Mitochondrial Activator of Caspase (SMAC/DIABLO) that modulates apoptosis by binding and activating certain inhibitors of apoptosis (IAPs). The anti-apoptotic activity of IAPs has been attributed, at least in part, to their ability to bind and inactivate caspases.

Many proteins may facilitates or inhibit apoptosis. The Bcl-2 family comprises proteins with structural homology that can mutually interact by forming heterodimers. While some members of the family, e.g. Bcl-2, prevent apoptosis, others, e.g. Bax, induce cell death (55). The expression level of these proteins plays an important role in determining the fate of the cell during toxic challenge. The key function of Bcl-2 family members seems to be the regulation of the release of pro-apoptotic factors from the mitochondria. Another family of proteins that can regulate apoptosis is heat shock proteins (HSPs). These proteins, which act as molecular chaperons in healthy cells, are induced by a variety of toxic stimuli, including heat, ischemia, toxins and heavy metals, and seem to play a role as defense mechanisms leading to cell survival. HSPs may participate in the refolding of damaged proteins and / or inhibition of apoptosis at different stages, up- or down-stream of mitochondrial events (56, 57).

As mentioned before, a critical role in signaling and perpetuation of apoptosis is played by caspases, a family of cysteine proteases that are present in cells as zymogens, requiring proteolytic processing to generate the two active subunits (58, 59). Caspases are divided into three groups, based on their specificity. The capability of certain caspases to cleave other member of the family creates the opportunity for sequential activation of these enzymes, resulting in a cascade of proteolytic events. Moreover, caspase activation causes degradation of cytoskeletal and nuclear proteins that are necessary for cell structures and cell survival (58-

60). Both cytoplasmic and nuclear changes occur during apoptosis, and a hallmark of apoptotic cell death is nuclear damage characterized by progressive DNA fragmentation into high molecular weight fragments and oligonucleosomal laddering. The biochemical process leading to DNA degradation is induced by nucleases, which may be dependent or independent from caspases (61, 62).

1.4.2 The resolution of apoptosis: The removal of apoptotic cells via phagocytosis is critical in preventing the spillage of intracellular contents with subsequent inflammatory response. For example, the phagocytosis of apoptotic neutrophils, which participate as a host defense at inflammatory sites to prevent injury and infection, is considered of major importance to avoid chronic inflammatory conditions (44). One major consequence of the deficiency to clear apoptotic cells may be autommunity (63), as observed in systemic lupus erythematosus, with production of autoantibodies against unengulfed apoptotic cells (64). Clearly, more knowledge about the mechanism of phagocytic clearance of apoptotic cells and the underlying regulatory machinery could have important implications for the development of therapeutic strategies for the treatment of chronic inflammatory and autoimmune disease. Theoretically, both enhancing and inhibiting apoptosis may protect against cell or tissue damage.

1.4.3 Role of apoptosis in UTI: Cell death through apoptosis has been documented in the course of renal injury both *in-vivo* and in *in-vitro* models (11, 65, 66). It has been shown that pyelonephritogenic *E.coli* diffusely adhere and promote cell lysis and apoptosis in epithelial cells (67). Clearance of inflammatory cells by apoptosis contribute to resolution of renal inflammation, and failure of this clearance may contribute to the persistence of the inflammatory process (68). There is general notion that apoptosis does not generate inflammation. However, it has been suggested that caspase-mediated apoptosis induced by *E.coli* bacteria may result in the shedding of urothelial cells together with bacteria in response to bladder infection (31). Inhibition of apoptosis resulted in decreased renal inflammation following ischemia-reperfusion (69). Also mononuclear cells infiltrate frequently accompanies local apoptosis. Therefore, the relationship between apoptosis and inflammation should be reevaluated. Apoptosis itself might promote inflammation through two mechanisms: disintegration of apoptotic cells with release of no-specific pro-inflammatory factors due to failure of the recognition/ engulfing mechanism; and active release of pro-inflammatory cytokines (68). It should be noted that altered expression of apoptosis-related protein such as Bcl-2, Fas and FasL results in autoimmunity and renal damage. If apoptotic cells are not adequately cleared, their contents might be released leading to an autoimmune response.

Clinical follow-up studies about apoptosis and renal scar in UTI are lacking, so any conclusion on the consequence of apoptosis in clinical settings remains speculative. Thus, although incomplete, the available in vitro and in vivo data suggest that apoptosis and its modulators (Bax and Bcl-2) contribute to the progress of renal tubular atrophy, cell damage and renal scarring (70). Understanding the role and regulation of apoptosis in renal infection has the potentials to provide the basis for the design of new diagnostic strategies as well as to improve current therapies (71, 72).

1.5 Anti-cell death machinery

The cell microenvironment usually contains multiple survival factors. The potential interaction of survival factors with lethal factors varies in a stimulus- and cell- specific manner. Cell death is usually a response to the cell microenvironment, where the absence of certain factors (survival factors) or the presence of lethal factors promotes apoptosis. Surrounding cells, soluble mediators and the extracellular matrix can affect cell death and survival. To counteract death signals, survival factors have been likened to efficiently inhibit a default apoptotic pathway that is ready to be executed (73-75).

Lysophosphatidic acid (LPA) is a natural lysophospholipid with high affinity to serum albumin. LPA is produced by platelets and has been shown to protect against apoptosis in a number of cell types including ovarian cancer cells, intestinal epithelial cells, hepatocytes, renal proximal tubular cells etc. The primary signaling mechanism that appears to be involved in this protection includes Gi, PI3K, Akt, and eNOS (75, 76). It is now clear that most responses to LPA are mediated via activation of GPCRs. Four LPA receptors have been identified to date and they are named LPA1, LPA 2, LPA 3 and LPA 4. While some GPCRs subtypes show selectively in their interaction with particular G proteins, LPA receptor subtypes appear to couple to multiple heterotrimeric G proteins. Specific LPA receptor subtypes have been implicated in activation pathways that protect against apoptosis in some cell types. It is likely that LPA plays a role as a local growth factor in response to cell injury, wound healing and inflammation, and may be part of a cascade that is triggered by invading bacteria during urinary tract infection. LPA induced protection against apoptosis has also been demonstrated to occur in vivo. In renal ischemia reperfusion injury model, LPA inhibited apoptosis of tubular epithelial cells, as well as renal expression of TNF-α and influx of neutrophils, both markers of inflammation (77).

Other studies have suggested that in the area of inflammation, nitric oxide (NO) seems to have a dichotomous function as both beneficial and detrimental molecule in the process of bacterial infection (36). One putative mediator of the NO-protective effects is heme oxygenase-1 (HO-1), which has been suggested to protect against apoptosis in renal cells (78, 79). The heme oxygenase (HO) enzyme system catalyzes the rate-limiting step in heme degradation, producing equimolar quantities of biliverdin, iron, and carbon monoxide (CO) (80). Induction of HO-1 is an adaptive and beneficial response to acute renal injury secondary to ischemia-reperfusion injury (81), nephrotoxins (e.g., cisplatin) (82) and glomerulonephritis (83) etc. Modulation of HO-1 expression using chemical inducers, inhibitors, and HO-1 gene delivery also support a functional role for HO-1 expression in ischemia-reperfusion injury in the liver, brain, and heart. The relevance of HO-1 expression is further substantiated by the presence of HO-1 protein in human renal tubules after ischemic acute tubular necrosis (84). The protective effects of HO-1 are mediated through one or more of several potential mechanisms. Increased HO-1 activity results in degradation of the heme moiety, a toxic prooxidant (85). The reaction also results in the generation of bilirubin, an antioxidant that is capable of scavenging peroxy radicals, inhibits lipid peroxidation, and has recently been shown to protect cells from a 10,000-fold excess of hydrogen peroxide (86). In essence, the induction of HO-1 results in shift of cellular redox toward a more antioxidant state rather than a pro-oxidant milieu. HO-1 induction has been associated with increased iron efflux, and the latter has been suggested as a mechanism for the cytoprotective effects of HO-1 (87). In addition, CO, the gaseous product, has vasodilatory effects similar to those of nitric oxide (NO) (88), as well as antiapoptotic and cytoprotective functions (89-91). Moreover, CO released in the process of heme degradation can inactivate existing iNOS-mediated damages by interacting with its heme iron moiety (92). Recent studies have also demonstrated an important role for the cell cycle regulatory protein p21 in mediating the protective effects of HO-1 expression in cell injury (79) and suggested that an appropriate level of HO-1 induction is beneficial, whereas too much HO-1 may, in fact, be a perpetrator of tissue injury (93).

Optimal levels of HO-1 are critical to determine whether the ultimate effect is one of protection or worsening the tissue injury. By deciphering the underlying molecular mechanism that controls the level of HO-1 enzyme activity, it will be possible to fine-tune HO-1 gene expression in disease states and exploit its use as a therapeutic strategy in the pathophysiology of renal injury. However, whether renal proximal tubular cells possess systems that can be rapidly recruited to defend against iNOS-mediated toxic effect of *E.coli* remains to be evaluated.

1.6 Effects of fetal development on cell death: There is a balance among a numbers of proteins that regulate cells survival/death. Therefore, any alteration in cell phenotype altering this balance may render the cell more sensitive or more resistant to apoptosis. During infancy and childhood, the body undergoes a process of maturation where cell phenotype changes progressively until the mature adult phenotype is achieved. This developmental process can be modified by events occurring early in life, i.e. according to the "programming hypothesis" (94). There is substantial evidence indicating that some diseases of adult life may arise from early events occurring in the prenatal period or in infancy. Several epidemiological studies have indicated that low birth weight infants have higher risk for cardiovascular and neurological disorders. It is therefore likely to speculate that fetal growth retardation may induce phenotypic alterations in some organs or cell types. *E.coli* infection retards renal growth in young rats due to inhibition of cell proliferation and activation of apoptosis (95, 96), indicating that the renal cortical cells of young animals are particularly sensitive to some toxic agents released by the bacteria. We have previously indicated that also neurons from fetal growth-retarded rats are more sensitive to apoptotic agents such as free radicals, probably due to a lower level of the defensive enzyme, catalase (97). These data may indicate that balance between pro- and anti-cell death machinery is altered. Therefore, it is worth to clarify whether renal cells from fetal growth retarded animals are more susceptible to different *E.coli* toxins.

2. AIMS OF THE PRESENT STUDY

The aim of the present study was to investigate the mechanisms that regulate renal cell survival and death during urinary tract infections.

The specific objectives were:

A. To better characterize the mechanisms by which uropathogenic *E.coli* toxins induce cell death in renal tubular cells.

B. To evaluate the role of nitric oxide in renal apoptosis induced by *E.coli* toxins.

C. To study the protective role of heme oxygenase-1 in *E.coli* toxins-induced apoptosis.

D. To evaluate whether fetal growth retardation permanently impairs the development of kidney, making renal cells more sensitive to bacterial toxin-induced apoptosis**.**

E. To identify *E.coli*-toxins that can induce renal proximal tubular cell death.

3. MATERIALS AND METHODS

3.1 Materials

A detailed description of the materials, including the list of antibodies and their working dilution, can be found in the methodological sections of the papers.

3.2 Cell culture and treatment procedures (paper I, II, IV)

LLC-PK1 cell line from porcine proximal tubule (European Collection of Animal Cell Cultures, ECACC) was maintained in medium 199 (M199) containing penicillin (50 U/ml) and streptomycin (50 μg/ml) and supplemented with 10% fetal calf serum (FCS). Cells were grown to confluence (untreated), then kept in 0.2% FCS medium for 4 h. Soluble toxins from uropathogenic *E. coli* strains or non-pathogenic strains were added at a final dose of 50 - 200 μl/ml. Cells left in 0.2% FCS medium were the control group (control, con). Cells were then collected for different assays by scraping or trypsinization. Albumin (1 mg/ml), lipid-free albumin (1 mg/ml), LPA (30 μM), L-NAME (10mM), D-NAME (10mM), ZnPP (2.5 - 10μM) and SNP (0.25 – 1 mM) were added to the cell culture just before adding the bacteria soluble toxins. Pertusis toxin (PTX, 200 ng/ml) was added 6 h before other substances. Wortmannin (Wort, 200 nM), MEK1/2 inhibitors PD98059 (PD, 50 μM) and U0126 (U0, 20 μM), cycloheximide (4μM), a guanylate cyclase inhibitor (ODQ 10 – 50 μM), hemin (10 - 100 μM) and a p53 inhibitor, pifithrin-α (PFT, 10 - 20μM) were added 30 min before bacterial soluble toxins. zVAD-fmk (35 μM) was added to the cell culture 20 h after the bacterial soluble toxins. We performed dose-response curves for all the above chemicals.

3.3 Bacterial strains (paper I, II, III, IV)

The pyelonephritogenic *E.coli* strain ARD6 strain, serotype O6K13H1 (98) was used in this study. Some of the common uropathogenic virulence factors have been found in *E.coli* ARD6, including CNF1, α-hemolysin, Type 1C pili and Afimbrial adhesin; Type 1 (fim A) and P-fimbriae were not detected. This strain impairs renal growth after pyelonephritis in young rats (96) and stimulates intensive inflammatory response in renal proximal tubular cells (13). A nonpathogenic *E. coli* strain W3110 (99) was compared. W3110 harboring plasmids pANN202-812 (100) was grown in agar-plats in presence of ampicillin (100 μg / ml). α-hemolysin was purified and assayed for hemolytic activity as described (101). Hemolysin

production by each isolate was determined by inoculation of that isolate onto 5% sheep-blood agar. Isolates exhibiting clearing around or beneath bacterial colonies after 18 h of incubation were considered to be positive for hemolysin (102).

In addition, we collected 67 *E coli* strains isolated from children admitted to our Emergency Unit with clinical symptoms of acute *E coli* pyelonephritis (fever≥38.5^{0}C, CRP≥ 20g/liter, *E coli* colonies in urine culture ≥ 10^5 CFU/ml). Five fecal *E.coli* strains were isolated from specimens submitted for investigation of intestinal pathogens. Ethical permission number is S439/01.

3.4 Preparation of bacterial soluble toxins (paper I, II, III, IV)

E.coli ARD6 (O6) was grown on LB-agar plates; one colony of bacteria was cultured overnight in 10 ml Luria-Bertani (LB) medium at 37°C until optical density reached 1.2. Overnight broth culture of *E.coli* was centrifuged (13,000 rpm, 5 min, 4ºC), and supernatant was filtered through 0.22-μm -pore-size filters. Total-protein concentration in the bacterial supernatant was determined using Bicinchoninic acid assay (Pierce, Rockford, Ill) in 100 x concentrated supernatant. In preliminary experiments, we found that total protein concentration was very similar among different original overnight cultures (ranging between 0.04 – 0.09 μg/μl).

3.5 Analysis of bacterial toxins (paper IV)

O6 supernatant was concentrated 100 times using 10,000-molecular weight-cutoff Miniplus Concentrator (Millipore). 100 μl of concentrated supernatant was injected into a Liquid Chromatography Controller LCC-500 PLUS (FPLC system, Pharmacia, Sweden) and 30 fractions were eluted incessantly at a flow rate 1mL/min. Different doses of the fractions were added to cell culture and cell viability was assayed by phase contrast microscopy and trypan blue exclusion test. The only one fraction that induced apoptosis was denatured, analyzed on SDS-PAGE and stained with 0.125% Coomassie brilliant blue to localize the exact size of the toxins. Protein sequences were identified by mass spectrometry of digests of isolated bands from the SDS-PAGE.

3.6 Analysis of cell morphology and viability (paper I, II, III, IV)

Cell morphological changes were routinely checked under phase contrast microscopy. Cell viability was assayed using the trypan blue (T.B.) exclusion test as previously described (103,

104). Cells with damaged cell membranes were stained blue (necrotic or late apoptotic cells), while cells with intact plasma membranes remained unstained (apoptotic and healthy cells).

3.7 MTT assay (paper I, IV)

MTT assay was based on the ability of mitochondria from viable cells to cleave the tetrazolium rings of the pale yellow MTT (3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide) resulting in formation of a dark blue formazan product. After removal of medium from 24-well plates, the cells were incubated with MTT. The MTT formazan precipitate was dissolved and read under a microplate reader (Dynatech, Chantilly, VA) with a test wavelength of 562 nm and a reference wavelength of 660 nm. The experiments were run at least three times in triplicates.

3.8 Annexin-V/propidium iodide (PI) staining (paper I, II, IV)

Early apoptotic cells were identified by Annexin V-fluorescein isothiocyanate (FITC) detection kit (Annexin Alexa, Molecular Probe). Cells on cover slips were washed in Annexin-binding buffer; then 3µl/100µl of Annexin V-FITC and 0.1µg/100µl of PI were added for 15 min in the dark at room temperature. The cells were examined under fluorescent microscopy (Nikon, Japan). Apoptotic cells were stained green and necrotic cell nuclei were stained red.

3.9 Immunocytochemistry (paper I)

The cells were grown on cover slips, washed with PBS, fixed with 3.7% formaldehyde in PBS for 15 min at 4°C and washed with 0.1% Tween-20 in PBS. Then the cells were incubated with a mouse monoclonal antibody (1:100 dilution in 1% BSA and 0.1% Tween-20 in PBS) against cleaved CK 18 (CK18 clone M30, Boehringer Mannheim) overnight at 4°C. After washing with PBS, the cells were incubated with a secondary anti-mouse-fluorescein antibody (1:500 dilution in 1% BSA and 0.1% Tween-20 in PBS) at room temperature for 2 h. The cover slips were mounted on slides with anti-fade mounting solution and examined under fluorescent microscope.

3.10 Immunoblotting (paper I, II, III, IV)

Cells were lysed in urea sample buffer (62.5 mM Tris-HCl, pH 6.8; 6 M urea; 10% glycerol; 2% SDS; 0.00125% bromophenol blue; 5% β-mercaptoethanol), sonicated for 15 s and then

incubated at 65°C for 15 minutes. PARP was detected with anti-PARP antibody (Santa Cruz, CA). ERK1/2 activity and total ERK were determined with anti-phospho-ERK1/2 and anti-total ERK antibody (New England BioLabs, Beverly, MA). Fas Receptor (FasR) was examined by anti-Fas antibody (Santa Cruz Biotechnology, CA). iNOS and eNOS were examined by an anti-iNOS/eNOS antibody (Stressgen Biotechnologies, Victoria, BC, Canada). HO-1 was checked with a rabbit anti-HO-1 antibody (Stressgen Biotechnologies). p53 and p21 were detected by a mouse monoclonal anti-p53 and anti-p21antibodies (Santa Cruz Biotechnology, CA).

3.11 Caspase activity assay (paper I, III)

Caspases are divided into 3 classes, based on the substrate-specificity. In these studies, we used a fluorogenic assay, which determines the cleavage of the substrate DEVD-MCA to evaluate the activity of class II caspase (2, 3 and 7). Cells were removed by gentle scraping. The capability of cell extracts to cleave the substrate DEVD-MCA, leading to release of free 4-methyl-coumaryl-7-amide (MCA, excitation 355 nm, emission 460 nm) was monitored at 37°C using Fluoroskan II (Labsystem AB, Stockholm). Fluorescent units were converted to pmoles 4-methyl-coumaryl-7-amide and subsequently related to protein content. Caspase 3-like activity was expressed in pmol/min/μg protein.

3.12 Detection of DNA fragmentation (paper I)

Internucleosomal DNA fragments were detected primarily by DNA laddering. Briefly, cells from 6-well plates were pelleted and lysed in 500 μl of lysis buffer (5mM Tris-HCL, pH 8.0, 20mM EDTA and 0.5% triton X-100), then centrifuged at 15000 rpm 15 min. The supernatant was precipitated with ethanol and 5 M NaCl overnight at –20°C. The samples were centrifuged at 15000 rpm for 15 min again and supernatant was discarded. The chromosomal DNA was extracted with RNAase and protease K (1mg/ml) for 1 h at 37°C, respectively. The DNA samples were separated by 1.8% agarose gel electrophoresis followed by staining with ethidium bromide.

3.13 Small interfering RNA (siRNA) (paper II)

LLCPK1 cells were transfected with 4 μg each of various expression plasmids by using Lipofectamine Plus (Invitrogen Life Technologies, Carlsbad, CA) in accordance with the manufacturer's protocols and used at 96 h after transfection, resulting in approximately 80%

transfection efficiency, as detected by fluorescent microscopy and flow cytometry. Vector constructs pSUPER-p53 for knockdown of p53, was kindly provided by Reuven Agami (105).

3.14 Animals (paper III)

Sprague–Dawley rats were kept in air-conditioned quarters, with a controlled photoperiod and free access to food and tap water. Procedures used in animal experimentation comply with the Karolinska Institute guidelines in the care and use of laboratory animals. For the prenatal exposure, pregnant rats (B&K, Stockholm, Sweden) were injected with dexamethasone (DEX) (Merck Sharp & Dohme, 0.1 mg/kg body weight per day i.p.) or vehicle from day 1 of pregnancy until parturition. As previously reported, prenatal DEX treatment results in 20–25% reduction in fetal growth, without affecting litter size or gestational length. This study was approved by the local ethical committee (N202/00).

3.15 Preparation of primary proximal tubular cells and treatment (paper III)

We used kidneys from 20-day-old Sprague±Dawley rats to prepare proximal tubule cells, as previously described (106). Cells were cultured in supplemented DMEM (20mM Hepes, 24mM NaHCO3, 10mgml-1 penicillin, 10 mgml-1 streptomycin and 10% fetal bovine serum) on 6-well plates for 96 h in 5% CO_2 at 37^0C. We found no morphological difference between cells from control-group (CON-cells) and cells from dexamethasone-treated-rats (DEX-cells). Cells were then kept in 1% serum medium (untreated group) for 4 h. Soluble toxins from uropathogenic E. coli ARD6 were added at a final dose of 100 µl/ml. Untreated group was left in 1% FCS medium until cells were assayed.

3.16 Measurements of catalase activity (paper III)

The activity of catalase was measured in renal cortex from CON- and DEX-treated rats of 20-day. Quantification of catalase activity was based on the decomposition of H_2O_2 as described by Aebi (107). Renal tissues were washed in PBS, the cortex was dissected and sonicated while kept on ice. Total protein concentration was determined by using Bio-Rad kit. The reaction was started by the addition of 5 µg total protein to PBS (1:100 vol / vol) containing 10 mM H_2O_2. Catalase activity was measured as the rate of disappearance of H_2O_2 during 30 s by monitoring absorbance at 240 nm. To determine the specification of the reaction, catalase activity was inhibited by the addition of azide.

3.17 Carbon monoxide exposure procedures (paper IV)

The cell culture flasks/plates were kept in the incubator at 37°C for 24 h before exposure to the CO gas. The CO-exposure chamber was kept constant at homogenous temperature and gas mixture concentration. The gas flow rate was primarily determined by the airflow; only the CO flow was adjusted to generate the different concentrations. CO level was measured throughout the experiments with a CO Electrochemical sensor (CO LS-6809620, Dräger Sicherheitstechnik GmbH, Germany). The pressure was controlled by a pressure regulator and flow controller (Brooks Instruments (Brooks Instrument, dev. of Emerson Process Management, USA). The percentage of oxygen (O_2) in the chamber was constant (20%) during the entire experimental procedure. O_2 concentration in the chamber was determined with O2 measuring spectrometer (Pac III) (Dräger Sicherheitstechnik GmbH, Germany). Since the partial pressure in the medium is low, Henry's law ($XCO = pCO/H$) is used to calculate the CO concentration in the fluid (108). XCO is the mole fraction of CO dissolved in the liquid phase, pCO is the partial pressure in the gas and H is Henry's constant (109). Cells were exposed to 1000 ppm CO in the air and therefore the CO concentration in the culture medium was calculated to be 1 μM. This is in agreement with the estimates of Thom et al (89) obtaining a CO concentration of 0.01-0.11 μM in a cell culture medium subjected to 10-100 ppm CO at atmospheric pressure. In preliminary experiments, we found that CO concentration as low as 1μM in the medium may reach the maximal protective effects. Thereafter 1 μM CO in the medium was used throughout all experiments. Cells in 0.2% FCS were exposed to CO in presence or absence of O6-toxins. During exposure to CO, the culture dishes were kept open. Untreated cells (no CO, no *E.coli* toxins) and O6-group (no CO, with *E.coli* toxins) were kept in otherwise identical condition in a separate incubator. Soluble toxins from uropathogenic *E. coli* ARD6 were added at a final dose of 100 μl/ml. 44 h after exposure to *E.coli* toxins, cells were then collected for different assays. Sodium nitroprusside, (SNP, 0.5 mM) and zinc protoporphyrin (ZnPP, 10μmol/L) were added to sets of cell culture just before adding *E.coli* toxins.

Results and discussion

1. Molecular mechanisms of apoptosis induced in renal proximal tubular cells by uropathogenic E.coli toxins. (Paper I, II).

Renal proximal tubular cell damages after urinary tract infection are mainly caused by *Escherichia coli (E.coli)*. Increasing number of studies has shown that *E.coli* and / or its toxins may stimulate apoptotic cell death in renal tubular cells, but the underlying molecular mechanisms remain to be elucidated. In a renal proximal tubular cell line (LLC-PK1 cells), we found that *E. coli* soluble toxins from the uropathogenic ARD6 (O6) strain induce apoptosis in a dose- and time- dependent manner. The expression of FasR and the phosphorylation of ERK1/2 were significantly upregulated by O6 soluble toxins in a time dependent manner. Cell death can be completely inhibited by two specific ERK1/2 inhibitors, but not by a broad caspase inhibitor, zVAD-fmk, implicating a caspase-independent pathway via ERK. These results indicate that during urinary tract infection, apoptosis induced by uropathogenic *E. coli*-toxins is dependent on ERK1/2. Caspases, although being activated, are not necessary for the cell death, and they act after the ERK signaling at which point cells become committed to cell death or can be rescued from death.

It has been suggested that renal tubular epithelial cells exposed to *E. coli* toxins may undergo cell death and that the inter- and intra-cellular regulator nitric oxide (NO) may play a role in the modulation of cellular viability in UTI. However, the role of NO pathway in renal proximal tubular cell death remains unclear. In the present studies, we have found that iNOS protein expression is upregulated when the cells are undergoing *E.coli* O6-toxins-elicited cell death. Given the known cytotoxicity of NO, it is likely that the reduction of NO production by suppression of NOS activity is necessary for the survival of the tubule cells. In agreement, we observed that, using specific NOS inhibitor, cell death is completely prevented. Similar protective effects of NOS inhibitor also have been shown in other models of renal damage (110). Furthermore, we demonstrate that, while the phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2) and iNOS protein level are upregulated in *Escherichia coli* (O6)-mediated death, the expression of eNOS is down-regulated. Up-regulation of iNOS by O6-toxins is regulated by ERK since blocking the iNOS upstream signal, ERK, reduces the level of expression of iNOS protein, and cell death is completely prevented. These results are consistent with those of other studies showing that ERK may modulate NOS-mediated death (111). Moreover, we observed that knocking down p53 does not rescue the cells, although p53 protein

is upregulated in O6-exposed cell, indicating that O6 toxins-iNOS-mediated cell death is regulated by ERK independently of the p53 pathway.

2. Activation of intrinsic molecules mediate survival signalling against cell death (paper I, II)

Counteracting death signals, survival factors have been likened to efficiently inhibit a default apoptotic pathway that is ready to be executed (73, 75). Lysophosphatidic acid (LPA) is a natural lysophoslipid with high affinity to serum albumin. In our model, we show that lysophosphatidic acid could trigger a survival signal through G-proteins and PI3K, consistent with LPA as a survival factor protecting against cell death (75, 76). It has been hypothesized that cell proliferation occurs in regions where tubular injury had occurred and local factors released in response to cellular injury may mediate nephron repair (112). It is likely that LPA plays a role as local growth or survival factor in response to cell injury, wound healing, and inflammation, and may be part of a cascade that is triggered by invading bacteria in UTI. Therefore, renal tubular epithelial cells exposed to *E. coli* toxins may be protected from cell death by local survival factors released by platelets or injured cells. The balance between pro- and anti-apoptotic factors may have relevance for the cell damage.

On the other hand, NO may mediate both toxic and protective effects in the process of bacteria infection (36). In our study, we have demonstrated that exogenous NO form donor SNP has dual effects on renal proximal tubular cells, i.e, low concentrations of NO protect cells against O6-toxins-induced death by activating HO-1, whereas high concentration of NO shows toxic action by inducing apoptosis. The toxic effect of *E.coli* toxins can also be abolished when HO-1 is activated by the specific HO-1 activator hemin. In presence of the specific HO-1 inhibitor ZnPP, the cells infected by *E.coli* toxins cannot be rescued by SNP or hemin. Necrosis observed here after inhibition of HO by ZnPP might be due to increased production of toxic oxidants as has been shown in other cell types (113). The anti-apoptotic actions of HO-1 induced by SNP in our model are in agreement with other studies showing that HO-1 activation attenuates iNOS-mediated toxicity by negatively modulating the expression or the activity of iNOS (92). Future therapeutic strategies aimed at manipulation of iNOS might reduce tubular cell shedding after *E.coli* toxins-induced injury in UTIs

3. Protective role of carbon monoxide in cell death (Paper IV)

HO-1 metabolizes heme into carbon monoxide (CO), biliverdin and iron (80, 93). CO released in the process of heme catabolism can inactivate existing iNOS by interacting with

its iron moiety. The current assumption is that while renal tubular cells undergo *E.coli*-toxins-induced cell death, HO-1 may activate cytoprotective effects that are associated with different end products of heme metabolism. One such product, CO, exerts potent cytoprotective effects, including reduction of inflammation and suppression of cell death, which are both mediated by p21 (114). In the present study, we provided direct evidence that CO may also play a role in reducing apoptosis in renal proximal tubular cells when infected by E.coli toxins. Although different CO concentrations (ranging from 0.1 μM to 10 μM) have been demonstrated to have cytoprotective and anti-inflammatory functions in vivo and in vitro experiments (89, 90, 115), the physiological concentration of CO in kidney is still unclear. In our experimental settings, the cells are exposed to 1 μM CO, and CO alone does not cause toxic effects, indicating that CO at certain range of concentrations could exert beneficial effects in kidney. Whether the concentration of 1μM CO we used in the present study to prevent cell death is within the physiological range needs to be further investigated. However, there are evidences showing that CO produces relaxation of rat-tail artery with a threshold concentration as low as 1μM (115) and that brain tissue in culture produce CO concentrations of the same magnitude as those used in vitro experiments (1 – 200 μM).

There is evidence that the cytoprotective effects of CO are mediated by p21 in other cell types (114). The anti-apoptotic effects of p21 are due to cell cycle arrest and inhibitory action on effector pathways for apoptosis (116, 117). We also found that p21 expression is up-regulated by CO concomitantly with cellular resistance to O6-toxins-induced death. Furthermore, we observed that the upregulation of p21 is not correlated with the expression of p53 that is an upstream molecule of p21 signaling, indicating that p21 gene is activated by p53-independent mechanisms. In conclusion, generation of CO elicited by HO-1 could promote survival signaling in renal cells. CO, therefore, might be a beneficial modality in renal cell response, perhaps in preventing cell shedding in UTI.

4. Effects of fetal growth retardation on renal apoptosis after UTI (Paper III).

Prenatal exposure to excessive glucocorticoids may alter the developing fetus inducing metabolic and endocrine imbalance in various organs, including the kidney. Our study has evaluated whether prenatal exposure to high levels of glucocorticoids adversely affects renal cell survival and predispose to renal cell death. Pregnant rats were injected with dexamethasone (DEX) from the day 1 of gestation. Renal proximal tubular cells (PTCs) were prepared from 20-day-old offspring in DEX-group (DEX-cells) and control-group (CON-cells). After 4 days culture, cells were exposed to uropathogenic *E.coli* ARD6 toxins, at

concentrations known to induce apoptotic cell death. We found that cell death rate was significantly higher in DEX- than in CON-cells. Cells exhibited morphological and biochemical features of apoptosis. Conversely, the activity of the antioxidant enzyme catalase was significantly increased in renal cortex homogenate from 20-day-old DEX-rats. No significant difference of cell death induced by H_2O_2 (1mM) between DEX - and CON-cells, and addition of vitamin E (50 μg/mL) did not prevent ARD6-induced cell death. These results indicate oxidative stress seems not to be of relevance in ARD6 toxins-induced cell death.

Therefore, prenatal exposure to high levels of glucocorticoids induces alterations in renal PTCs rendering them more sensitive to *E.coli* toxins via non-oxidative stress. With the increasing use of multiple doses of glucocorticoids in preterm infants, the possibility that antenatal glucocorticoids may lead to renal adverse consequences is of clinical relevance. At the present time, there is no clear-cut evidence of deleterious long-lasting effects of prenatal glucocorticoids on renal function in humans (118). However, Hellstrom and his co-workers demonstrated that fetal growth retardation (a situation often associated with high prenatal glucocorticoids levels) make the kidneys more prone to develop renal damage when infected in childhood (119). The role of prenatal glucocorticoids as a risk factor for scar development after urinary tract infection deserves therefore further attention.

5. Identification of the *E.coli* toxins responsible for apoptotic cell death (paper II, IV and personal observations)

Secreted proteins are virulence factors of many bacterial pathogens. A mechanism used by pyelonephritogenic strains to promote UTIs is by targeting proteins to extracytoplasmic region via a secretion system (9). Some virulence factors such as hemolysin (hly), cytotoxic necrotizing factor type 1 (CNF1) and LPS are produced by the O6 strain. These toxins may cause cytotoxic effects in certain type of cells (120). To evaluate whether any of these toxins may induce apoptosis in renal proximal tubular cells, we examined the potential effects of every single virulence factor respectively. After treatment, cells were studied by phase contrast microscopy, trypan blue exclusion assay, Annexin V / PI staining and Western blotting for PARP cleavage. Purified hly from O6 as described in (13), and commercial available hyl induce mainly necrosis at doses of 5×10^{-3} - 10^{-1} μg/ ml within 24 h. Moreover, supernatant from transformants of W3110 expressing hemolysin (W3110-hly+) stimulates both necrosis and apoptosis in a dose-dependent manner after 44 h exposure. At 44 h more than 40% apoptotic cells were observed, indicating that hemolysin may contribute to the pro-apoptotic mechanism in renal tubular cells during *E.coli* infection. Moreover, O6-supernatant in which LPS was

destroyed by periodate oxidation still could induce apoptosis, and LPS at doses of 50 – 500 ng/ml did not induce apoptosis. O6-supernatant pre-treated by protease K resulted in the loss of pro-apoptotic activity. CNF negative *E.coli* supernatant also induced the same type of cell death as O6 and the other CNF positive *E.coli* supernatant did.

To further confirm and characterize the pro-apoptotic toxins, we fractionated the O6 supernatant by using FPLC. Only one fraction (1 of 30) was able to induce apoptosis, whereas all other fractions did not induce cell death (at least in the concentration we tested). Moreover, we analyzed the fraction that contains the pro-apoptotic activity by 8% SDS-PAGE and stained the PAGE by Coomassie blue. We observed that there were several proteins with a molecular range 52 – 120 kDa, the main bands were localized at 82-120 kDa. By mass spectrometry of digests of isolated bands from the SDS-PAGE, we identified the protein sequences corresponding to three proteins, showing that the FPLC fraction contains hemolysin, heat shock protein 70 and unknown protein(s).

In order to evaluate whether this pro-apoptotic toxins are expressed in other *E. coli* strains, we tested *E.coli* strains isolated from the urine of 67 children (<2 years of age) admitted to our Emergency unit with clinical symptoms of pyelonephritis (positive urine culture, high fever, high CRP). We found that 21% strains expressed hemolysin and all of them induce apoptosis. 36% strains without hemolytic activity also induce apoptosis.

These results suggest that the pro-apoptotic *E coli* O6-toxins are proteins with MW of 80-120 kDa. Hemolysin is one of the toxins involved in triggering apoptotic machinery in renal proximal tubular cells. However, some hemolysin negative strains also induce the same type of cell death.

Summary

Response of renal cell to uropatogenic *E.coli* toxins

Soluble toxins from uropatogenic *E.coli* induce cell death in renal proximal tubular cells. The "checkpoint" for cell death requires activation of ERK, finally leading to activation of caspases, a group of cysteine proteases, which are responsible for the features of apoptosis by cleaving a set of proteins. E.coli induced-cell death is independent of p53, although p53 protein expression is upregulated. iNOS and FAS protein expression were upregulated when cells were exposed to *E.coli* toxins and meanwhile eNOS protein expression is down regulated. Interestingly, cell death could be inhibited with both NO donor and NOS inhibitor suggesting that iNOS is involved in *E.coli* toxins-mediated cell death.

Intrinsic survival signaling

In addition to "death molecules", cells can also express survival factors, which are regulated by cytokines and other death-survival signals at different levels. LPA is a normal constituent of serum and binds with high affinity to albumin while retaining its biological activity (76). LPA is released from platelets during infections. Cell death induced by *E.coli* can be inhibited by LPA via G-protein and PI3 kinase. The toxic effect of *E.coli* toxins can also be abolished when HO-1 is activated. The anti-apoptotic actions of induced HO-1 in our model are in agreement with other studies showing that HO-1 activation attenuates iNOS-mediated toxicity by negatively modulating the expression or the activity of iNOS (92).

Carbon monoxide in preventing cell death

The generation of CO elicited by HO-1 could promote survival signaling in renal proximal tubular cells. CO, therefore, might be a beneficial modality in renal cell response, perhaps in preventing cell shedding in UTI. Strategies to achieve regulated induction of CO might have therapeutic implications in several clinical settings involving the kidney. However, the endogenous concentration of CO, both in circulating and in tissue is unknown, but such knowledge is prerequisite to any design of future remedies targeting increasing endogenous CO production.

Fetal growth retardation and renal proximal tubular cell death

There is substantial evidence indicating that some diseases of adult life may arise from early events occurring in the prenatal period or in infancy. The present results clearly indicated that renal proximal tubular cells from fetal growth-retarded rats are more sensitive to apoptotic

agents such as *E.coli* toxins, which is likely due to no-oxidative stress. With the increasing use of multiple doses of glucocorticoids in preterm infants, the possibility that antenatal glucocorticoids may lead to renal adverse consequences is of clinical relevance.

Hemolysin is involved in inducing apoptosis in renal proximal tubular cells in UTI

Hemolysin is involved in triggering apoptotic machinery in renal proximal tubular cells. The occurrences of cells undergoing apoptosis have been evidenced in renal proximal tubular cells by exposure to both hly+ *E.coli* strains and non-pathogenic *E.coli* transformed with hly+ plasmid. Some hemolysin negative strains also induce the same type of cell death, suggesting that hemolysin is not the only one toxin that can induce renal tubular cell apoptosis during UTI. It is still of clinical relevance to identify those unknown toxins that may stimulate renal tubular cells apoptosis in UTI. However, the role of E.coli toxins-induced apoptosis in UTI and renal scarring is, to large extend, speculative. Further studies are needed to get more insight into this aspect.

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