CHAPTER 1

General introduction

CONTENTS

1.1 ISCHEMIA/REPERFUSION INJURY

Ischemia/reperfusion (I/R) is an inevitable and injurious event in clinical conditions such as infarction, sepsis and solid organ transplantation. Ischemia occurs after insufficient local blood supply leading to oxygen deprivation (i.e. hypoxia), accumulation of cellular waste, nutrient deprivation and an excess of carbon dioxide (i.e. hypercapnia). Depletion of cellular energy (ATP) is the most prominent cause of cellular injury during ischemia. Reperfusion of ischemic tissue e.g.. following transplantation provides oxygen as well as substrates that are necessary for tissue regeneration, restoration of energy levels and concurrent removal of toxic metabolites. Nevertheless, restoration of blood flow to ischemic tissue paradoxically exacerbates tissue damage by initiating a cascade of inflammatory events including release of reactive oxygen species (ROS), pro-inflammatory cytokines and chemokines, recruitment of leukocytes and activation of the complement system (1-4). Such deterioration of tissue function and integrity after reperfusion is defined as ischemia/reperfusion injury (IRI). The close interaction between many cell types and mediators involved in the pathophysiology of IRI complicates the treatment of this condition. To date, no effective therapy or treatment for IRI in the clinic exists.

1.1.1 Renal ischemia/reperfusion injury

Renal I/R is an inflammatory process that leads to acute kidney injury (AKI). AKI is a clinical syndrome characterized by a rapid (hours to days) decrease in renal function and accumulation of products of nitrogen metabolism in the plasma, such as creatinine and urea. Other common clinical manifestations include decreased urine output (oliguria), accumulation of metabolic acids and increased potassium and phosphate concentrations. AKI may not only occur in the context of kidney transplantation in which I/R is inevitable, but is also a consequence of impaired kidney perfusion e.g. during major surgery or sepsis. Incidence of AKI varies from more than 5000 cases per million people per year for non-dialysis-requiring AKI, to 295 cases per million people per year for dialysis-requiring disease (5). AKI has a frequency of 1,9% in hospitalized patients (6) and is especially common in critically ill patients, in whom the prevalence of AKI is greater than 40% at admission to the intensive-care unit if sepsis is present. Occurrence is more than 36% on the day after admission to an intensive-care unit (7), and prevalence is greater than 60% during intensive-care-unit admission (2). In the renal transplant setting, ischemia during the transplant procedure, under toxic therapautical conditions (calcineurin inhibitors) or immunological injury, affects viability and promotes alloimmunity. Therefore, AKI not only has a major impact on short-term but also on long-term graft survival following kidney

transplantation and is strongly associated with delayed graft function (DGF), clinical morbidity and mortality (8-12). In order to resolve the shortage of kidney donors, there is an increased use of marginal donors, including older and cardiac death donors (CDD). In contrast to organ donation from living or brain death donors, the delay between circulatory arrest and organ preservation in CDD causes additional ischemic injury in these organs. As a consequence, the incidence of DGF and primary nonfunction in CDD kidney transplantation is relatively high. Currently, approximately a quarter of all kidney transplantations in the Netherlands are performed using kidneys from CDD donors, and therefore an effective therapy for renal IRI is imperative.

1.1.2 Kidney anatomy

The human kidney (Fig 1A) contains approximately one million functional units (the nephrons; Fig 1B) that consist of a filter (the glomerulus) and a processing portion i.e. the proximal tubule, the distal tubule and collecting duct. The renal cortex is the outer portion of the kidney between the renal capsule and the renal medulla and is the part of the kidney where ultrafiltration takes place. The cortex includes the renal filters (glomeruli), Bowmans capsule and renal tubules.

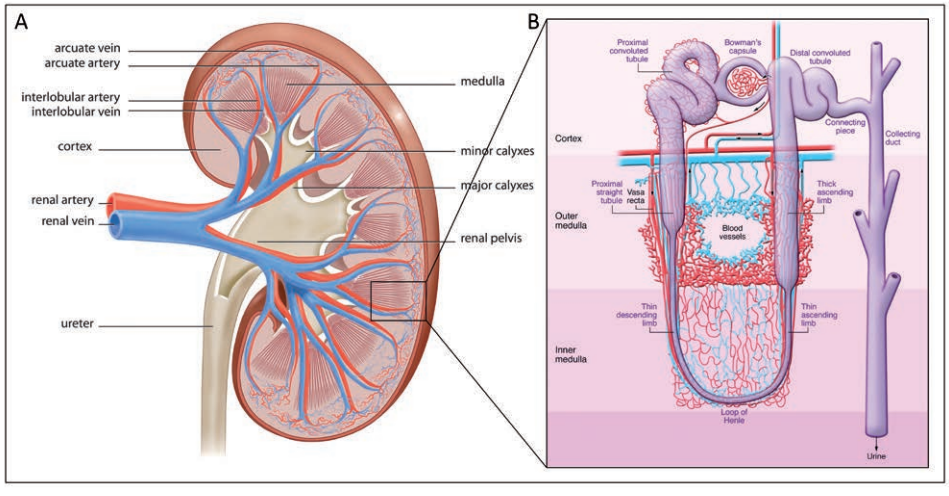

Figure 1. Normal kidney and nephron with medullary microvascular anatomy. Anatomy of the kidney (A) and a nephron (B) with regions identified. Outer medulla vasculature is shown with capillaries in red and venous system in blue. The vasa recta with countercurrent exchange of oxygen resulting in a gradient of decreasing oxygen tension. *Adapted by permission from Bonventre et al, J Clin Invest. 2011; copyright American Society for clinical investigation.*

The renal medulla is the innermost part of the kidney and contains the loops of Henle, which are responsible for maintaining and fine-tuning the salt and water balance of the blood. The renal medulla is made up of approximately seven

pyramids of which the apex or papilla points internally into the medulla. The renal papilla is the location, where the urine from the collecting ducts ends up in the calyses before it passes further into the urinary tract via the renal pelvis and ureter to the bladder.

The glomeruli produce approximately 180 liters of primary filtrate (pre-urine) every day of which only 1 to 2 liters are finally excreted as urine. Filtered metabolites in the primary filtrate are reabsorbed in the tubular structure, which is covered by a single layer of epithelial cells. These tubular cells are specialized in tubular reabsorption and are surrounded by peritubular capillaries (13). Tubular reabsorption is the process by which filtered metabolites e.g. salts, proteins and glucose are taken up from the primary filtrate and transported back into the blood via the tubular cells. Eighty percent of the renal oxygen consumption is utilized to drive the Na+-Ka+-ATPases on the basal side of the tubular cells, which are responsible for sodium reabsorption from the urine. The proximal tubular epithelial cells (PTEC) play an important role herein. The luminal surface of the epithelial cells of this segment of the nephron is covered with densely packed microvilli forming the brush border. These microvilli greatly increase the luminal surface area of the cells, facilitating their reabsorptive function (13). Because resaborption is a process with very high energy expenditure, PTEC are equipped with a vast amount of mitochondria and are highly dependent on oxidative phosphorylation.

In the cortex, the partial pressure of oxygen (PO2) is 50 mmHg, but only 10–20 mm Hg in the outer medulla, which harbors the S3 segment of the proximal tubule. This low oxygen-pressure is not only a consequence of the high metabolic requirements of the PTEC here, but is also due to the countercurrent arrangement of vessels that drain the outer and inner medulla (Fig 1B). As blood flows down toward the tip of the medulla, most of the oxygen diffuses out of the descending vasa recta into the interstitium, the space between the tubules. It can then either diffuse to the surrounding tubules, where it is consumed for active transport processes, or be reabsorbed into the ascending vasa recta and carried back to the cortex. Oxygenation of the outer medulla is therefore limited by the diffusional shunting of oxygen between descending and ascending vasa recta (14). Shortage of oxygen during and after renal ischemia therefore most profoundly affects PTEC in the S3 segment of the outer medulla, which have a high energy expenditure, but due to the countercurrent vessel arrangement a relatively low surrounding PO2.

1.1.3 Acute tubular necrosis

Ischemic AKI following renal I/R is characterized by injury to the PTEC in the S3 segment of the nephron in the outer medulla and cortico-medullary junction

(Fig 2). During an ischemic event, there is shedding of the proximal tubular brush border within several minutes (15) and loss of polarity with mislocalization of adhesion molecules, complement regulators and other membrane proteins like the Na+-Ka+-ATPase and integrins (16;17). Disruption of the cytoskeleton leads to a loss of tight and adherens junctions, which normally actively participate in function as paracellular transport, cell polarity, and cell morphology. Opening of tight junctions leads to an increased paracellular permeability and backleak of the glomerular filtrate from the lumen to the interstitium (18). Disruption of microvilli and their detachment from the apical cell surface leads to formation of membrane-bound blebs early following ischemia, which are released into the tubular lumen. In advanced ischemic injury, viable and necrotic tubular epithelial cells are desquamated, leaving the denuded basement membrane as the only barrier between the filtrate and the peritubular interstitium, resulting in even more backleak of glomerular filtrate (4;19;20). The sloughed tubular cells,

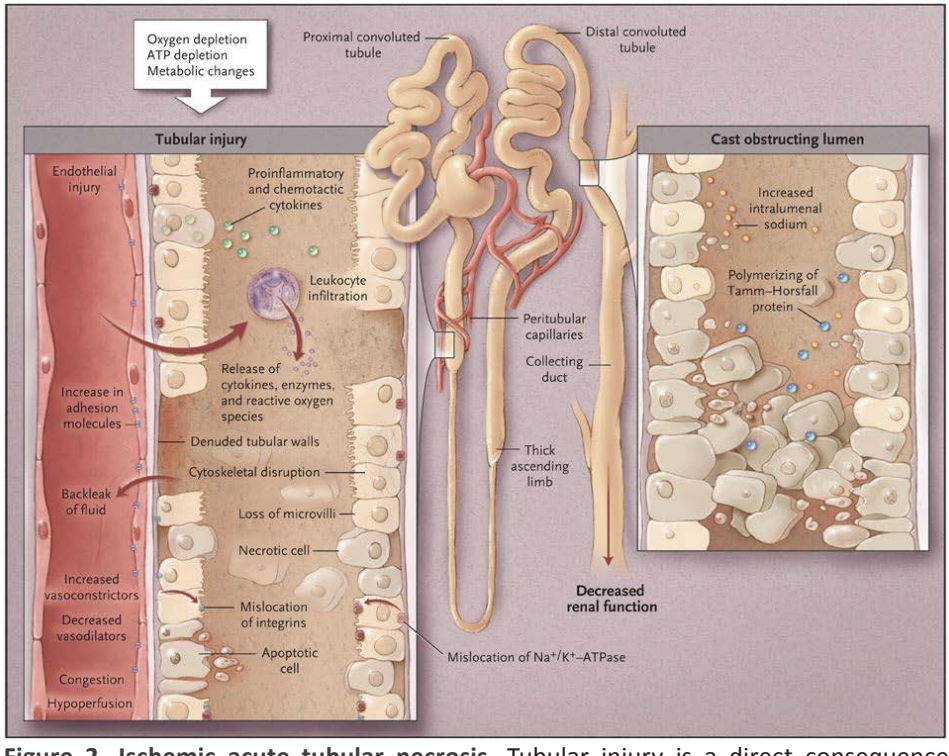

Figure 2. Ischemic acute tubular necrosis. Tubular injury is a direct consequence of metabolic pathways induced by ischemia but is potentiated by inflammation and microvascular compromise. Acute tubular necrosis is characterized by shedding of epithelial cells and denudation of the basement membrane in the proximal tubule, with backleak of filtrate and obstruction by sloughed tubular cells. *Reproduced with permission from Abuelo et al, N Engl J Med. 2007, Copyright Massachusetts Medical Society.*

brush-border vesicle remnants and cellular debris along with tamm-horsefall protein form characteristic tubular casts, which have the potential to obstruct the tubular lumen, thereby increasing intratubular pressure and preventing glomerular filtration in the affected nephron. Denuded basement membranes and casts obstructing tubules are therefore a hallmark of ischemic AKI (Fig 3). In addition, proximal tubular cell injury and dysfunction during ischemia/reperfusion results in afferent arteriolar vasoconstriction mediated by the tubuloglomerular feedback, luminal obstruction and backleak of filtrate leading to a persistant regional hypoxia and additional tubular injury, even when the kidney is reperfused (21;22).

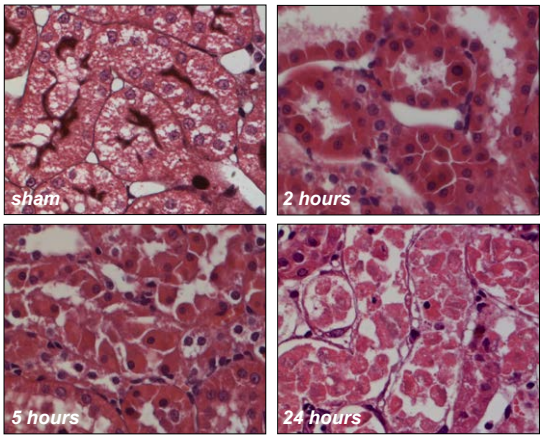

Figure 3. Ischemic acute tubular necrosis in the outer medullary region following I/R in rats. ATN is characterized by loss of the brush border and tight and adherens junctions between tubular epithelial cells (2h after I/R) followed by detachment from the basement membrane (5h after I/R). Basement membranes are completely denuded after 24h reperfusion and characteristic tubular casts of sloughed tubular cells are present in the lumen of the tubules.

1.1.4 Tubular injury markers

At present, the diagnosis of AKI is mainly based on measurement of serum creatinine and ureum levels. However, these traditional methods are not very sensitive and specific for the diagnosis of AKI, given that a 50% loss in renal function is required before creatinine levels rise (23), the method is dependent on nonrenal factors independent of kidney function (age, sex, muscle mass, infection) and several medications alter the tubular secretion of creatinine leading to changes in serum creatinine independent of glomerular filtration (24). AKI is associated with increased morbidity and mortality in critically ill patients and a quick detection is difficult with serum creatinine and ureum. A number of serum and urinary proteins have been identified that may detect AKI prior to a rise in ureum and serum creatinine. Promising new biomarkers of AKI are Neutrophil Gelatinase-Associated Lipocalin (NGAL) and Kidney-injury molecule (KIM)-1 (25).

NGAL is a 25 kDa protein firstly identified in specific granules of the neutrophil, where it was bound to gelatinase. NGAL is an important component of innate immunity to bacterial infection and is expressed by hepatocytes, immune cells

and renal tubular cells in various disease states (25;26). NGAL is highly resistant to proteolysis, which enhance the potential suitability as a clinical biomarker. In the kidney, NGAL is produced by tubular epithelial cells of the proximal and distal segment. In addition, circulating NGAL is freely filtered by the glomerulus and is undergoing rapid clearance by the proximal tubule via receptor binding and endocytosis. In healthy kidneys, NGAL is only detectable at low levels, however, in the setting of acute tubular injury, it undergoes rapid and profound upregulation with large increases in both urine and plasma. This rapid response enables NGAL to potentially identify injured kidneys much earlier than was previously possible using traditional markers of renal function such as creatinine. The functional role of NGAL has not been completely unraveled. It seems to be involved in iron transportation to and from the proximal tubular epithelial cells. Animal studies have demonstrated a renoprotective effect of exogenously administered NGAL in the setting of acute ischemic injury (27).

Kidney injury molecule-1 (KIM-1) is a putative epithelial cell adhesion molecule containing an immunoglobulin domain (28). KIM-1 mRNA and protein are expressed at a very low level in normal kidney, however in the setting of acute tubular injury (Fig 4), its expression increases dramatically in proximal tubular

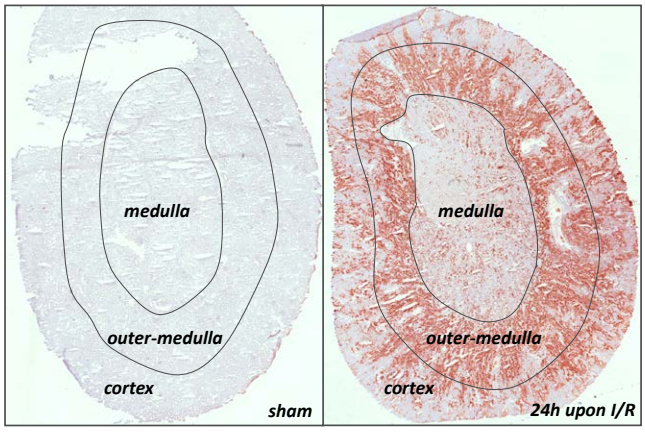

Figure 4. Kidney injury molecule (KIM)-1 after I/R. Whole rat kidney staining of KIM-1 in an untreated (sham) or clamped (45 min) kidney 24 hours after reperfusion. KIM-1 is most present in the outer medulla, which harbors the proximal tubular epithelial cells in the S3 segment of the nephron.

epithelial cells (29;30). KIM-1 has also been identified as the first nonmyeloid phosphatidylserine receptor that confers a phagocytic phenotype on injured proximal tubular epithelial cells both *in vivo* and *in vitro* (31). Similiar to NGAL, urinary KIM-1 has been found to be an early indicator of AKI that compares favorably to a number of conventional biomarkers and tubular enzymes (29;32).

1.1.5 Endothelial injury

Besides the tubular compartment of the kidney, the microvascular compartment is also critically involved in the pathophysiology of ischemic AKI, especially

during the reperfusion phase when leukocytes are able to interact with ischemic and injured endothelium (19). As stated earlier, blood flow to the outer medulla of the kidney is largely reduced following perfusion (33-36). This marked hypoperfusion of the outer medulla is persistent even though cortical blood flow improves during reperfusion after an ischemic insult. Small arterioles in kidneys following reperfusion vasoconstrict more than do vessels in normal kidneys (37;38). Enhanced vasoconstriction together with small vessel occlusion due to endothelial-leukocyte interactions and activation of the coagulation system result in local compromise of the microcirculation and regional ischemia, especially in the outer medulla further inducing tubular ischemic injury (39). Local blood flow to the outer medulla, already reduced due to arteriolar vasoconstriction, is further compromised by local edema. In addition, endothelial cells contributes to the pathology of IRI by enhanced endothelium-leukocyte interactions due to increased expression of cell adhesion molecules such as ICAM-1 on damaged endothelial cells combined with increased expression of counterreceptors on leukocytes (40). This results in activation of leukocytes, obstruction of capillaries, further activation and transmigration of leukocytes, production of cytokines, and an extensive proinflammatory state (39). Damage to the endothelium, loss of the glycocalyx, disruption of the endothelial cytoskeleton, breakdown of the perivascular matrix and alteration of endothelial cell-cell contacts all culminate in increased microvascular permeability during AKI and loss of fluid into the interstitium (19;41;42).

Renal I/R impairs the integrity of endothelial cells and leads to loss of peritubular capillaries (41;43-48). This reduced number of vessels is associated with chronic hypoxia (49), which can be expected to lead to increased tubular injury and tubulointerstitial fibrosis. Pericytes, also called perivascular fibroblasts, play a critical role in the stabilization and proliferation of peritubular capillaries via interaction with endothelial cells (50-52). Recent studies have shown that in renal I/R, pericytes detach from the endothelium and migrate to the interstitium to become activated and differentiate into myofibroblasts contributing to renal fibrosis (53;54). The critical stabilization of endothelial cells by pericytes is mediated by several angioregulatory factors, including the anti-inflammatory factor Ang-1 produced by pericytes and the pro-inflammatory factor Ang-2 produced by activated endothelial cells (52;55;56). Angiopoietins are a group of vascular regulatory molecules that bind to the receptor tyrosine kinase Tie-2, which is predominantly expressed by vascular endothelial cells. Ang-1 is a strong vascular protective agonist of the Tie-2 receptor responsible for preventing vascular leakage, maintaining endothelial cell survival and inhibiting vascular inflammation. Ang-2 acts as an antagonist of Ang-1 and in a dose dependent manner promotes destabilization, vessel leakage and inflammation. A dysbalance

towards Ang-2 will therefore lead to loosening contacts between endothelial cells and perivascular cells, with subsequent vessel destabilization and abnormal microvascular remodeling (55-57).

1.1.6 Stress and cell death mechanisms

During and following renal ischemia, several cellular stress mechanisms are activated to cope with nutrient and energy depletion, ROS formation and accumulation of toxic metabolites. During ischemia, ATP shortage caused by hypoxia and glucose deprivation decreases the calcium concentration in the endoplasmic reticulum (ER) of tubular cells, thereby impairing the activity of chaperone molecules and maturation of native proteins. The accumulation of excessive amounts of mis- and unfolded proteins in the ER following ischemia causes ER-stress, which results in activation of the so called ER-stress response or unfolded protein response (UPR) (58-60). The ER-stress response is aimed to adjust cell functions in response to ER-stress and to re-establish normal ER function both at the translational and transcriptional level.

Upon accumulation of unfolded proteins, 78-kDa glucose-regulated protein (GRP78 or BIP), which is one of the most abundant ER luminal chaperones (61;62), binds to unfolded proteins and thereby dissociates from the three membrane-bound ER-stress sensors (Fig 5). These stress sensors include pancreatic ER kinase (PKR)-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6). The dissociation of GRP78 from these stress sensors allows their subsequent autophosphorylation and activation.

Phosphorylated PERK decreases protein synthesis by phosphorylation of eIF2α. Phosphorylated IRE1 increases cellular degradation of unfolded proteins by splicing of transcription factor XBP-1, inducing expression of genes involved in ER-associated protein degradation (ERAD). Proteolytic cleaved ATF6, also a transcription factor, enhances the protein folding machinery by inducing the transcription of ER-chaperones. Thus, all three pathways are critical for handling ER stress and for return to normal homeostasis. The adaptive responses to the accumulation of un- or misfolded proteins in the ER provide initial protection from cell death. However, prolonged or excessive ER-stress can trigger cell death, classically through the process of apoptosis. ER-stress-induced apoptosis is mediated by mitochondria-dependent and -independent pathways (60;63).

In renal IRI several morphologically distinct cell death programs have been recognized including type I cell death (apoptosis), type II cell death (autophagy) and type III cell death (necrosis) (64-67). These death programs are often intertwined and depending on the energy status, signaling events and therapeutics applied can occur simultaneously or as a continuum. Even features of both apoptosis and necrosis may coexist in the same cell. Moreover, the process of autophagy

is used to engulf apoptotic or necrotic cells (68). In addition, if engulfment is absent, dead cells in the late stages of apoptosis may present necrotic features due to the loss of cellular energy and plasma membrane integrity. This process is called apoptotic necrosis or secondary necrosis (69). Collectively, these death programs following renal I/R are often, although mistaken, defined as acute tubular necrosis (ATN). Apoptosis (type I) is a regulated, genetically determined mechanism designed to dismantle cells systematically (e.g. cells that are no longer functionally viable) (70). Importantly, apoptosis is therefore an energy-dependent process and often cannot take place during severe ischemia. The process involves an orchestrated caspase signalling cascade that ultimately leads to cell rounding and shrinkage, chromatin condensation, DNA fragmentation, blebbing of the plasma membrane and nuclear fragmentation. Subsequently, the formed apoptotic bodies can be cleared effectively by phagocytes. Therefore immunogenic endogenous molecules are not released into the extracellular environment (69;71;72) and inflammation is prevented.

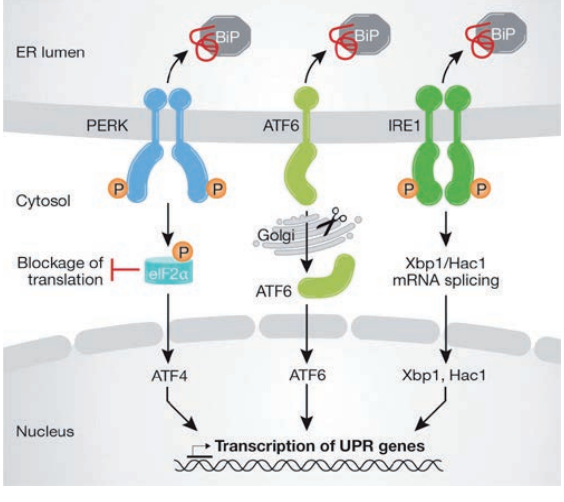

Figure 5. Stress sensors in the ER-stress response. Accumulation of mis- or unfolded proteins in the ER-lumen results in ER-stress and activation of the ER-stress or unfolded protein response (UPR). Release and binding of GRP78/BiP to mis- or unfolded proteins activate the ER-stress sensors PERK, ATF6 and IRE1 at the onset of ER stress. To re-establish homeostasis and normal ER function, the ER-stress response initiates a global decrease in protein synthesis, while increasing the production of ER-chaperone proteins and ER-associated degradation (ERAD). *Reprinted by permission from Macmillan Publishers Ltd: Cyr et al, EMBO reports (2009) 10, 1206 - 1210), copyright 2009.*

Autophagy is responsible for the degradation of cytoplasmic material, e.g. proteins and organelles, which are sequestered by intracellular double-membrane structures called autophagosomes. These autophagosomes then subsequently fuses with lysosomes resulting in proteolytic degradation yielding new macromolecules for the synthesis of vital cellular components. Autophagy occurs at a basal level in most cells and contributes to the turnover of long-lived proteins and organelles to maintain intracellular homeostasis. In response to cellular stress (e.g. ischemia), autophagy is up-regulated and can provide an

adaptive strategy for cell survival, but may also lead to autophagic cell death (type II) (69;71;72). Because of this dual role, it remains uncertain whether autophagy is a mechanism of cell death or survival in the pathophysiology of renal IRI (67;73;74).
During ischemia, necrosis (type III) takes place when insufficient ATP is available. The process involves cellular and organelle swelling, reactive oxygen species production and rupture of the plasma membrane. The processes might result in the release of intracellular molecules and danger-associated molecular patterns (DAMPs) that can elicit a sterile inflammatory response (69;71;72). Importantly, both late apoptotic as well as necrotic cells activate the complement system (75-78).

1.1.7 Inflammation

Depletion of cellular energy is the most prominent cause of tubular injury during ischemia. Nevertheless, reperfusion of ischemic tissue paradoxically exacerbates tissue damage by initiating a cascade of inflammatory events. For this reason, deterioration of tissue function following reperfusion is often defined as reperfusion-injury, however both the injury during the ischemic and reperfusion phase is included herein. DAMPs released during ischemic tissue injury, altered or enhanced expression of membrane-bound proteins and activation of endothelial and tubular cells during reperfusion collectively promote an inflammatory environment in which both innate and adaptive immunity are involved and contribute to the pathology of IRI (39). Innate immunity is responsible for the early response to injury in a non-antigen-specific fashion and comprises humoral components including the complement system as well as innate immune cells including neutrophils, macrophages and dendritic cells (DCs). In the renal transplant setting, adaptive immunity activated by specific alloantigens is initiated within hours, lasts over the course of several days after injury and includes DC maturation and alloantigen presentation, and T- and B-lymphocyte proliferation and activation.
Tubular cells themselves also actively participate in the inflammatory response in renal IRI (79;80). In addition to generating proinflammatory and chemotactic cytokines such as TNF-α, MCP-1,IL-8,IL-6,IL-1β and RANTES which activate inflammatory cells, tubular cells also express Toll-like receptors (TLRs), complement proteins and receptors (79;80), and costimulatory molecules, which regulate T-lymphocyte activity. Endogenous ligands released from damaged and stressed tissue signal through TLRs on tubular cells (81;82). These ligands include heat-shock proteins (binding to TLR2 and -4), the non-histone chromatin-binding protein high-mobility group box 1 (HMGB1) (TLR2 and -4), and ECM components such as hyaluronan (TLR2 and -4), fibronectin (TLR4), heparan sulfate (TLR4),

and biglycan (TLR2 and -4) (83-88). Activation of TLR-2 and -4 on tubular cells initiates a proinflammatory response marked by the release of cytokines and chemokines, which attract inflammatory cells. In addition, tubular cells express MHCII and costimulatory molecules and can activate T-cells (80;89-91).
Neutrophils interacting with the activated endothelium, infiltrate into the interstitium and there excrete reactive oxygen species, proteases, myeloperoxidase and cytokines. These events lead to increased vascular permeability and reduced tubular epithelial and endothelial cell integrity (92), aggravating kidney injury (93).
One of the major cell types that accumulates around tubules after I/R is the macrophage (94). Proinflammatory (M1) macrophages are recruited into the kidney in the first 48 hours after I/R, whereas mannose receptor–positive, non-inflammatory (M2) macrophages predominate at later time points. Depletion of macrophages before I/R has been shown to diminish kidney injury, whereas depletion at several days after injury slows tubular cell proliferation and repair, indicating a switch from a proinflammatory to a trophic macrophage phenotype that supports the transition from tubule injury to tubule repair (95).
In addition to macrophages and DCs, also T-lymphocytes infiltrate into the kidney in both the early and later phases of AKI and can facilitate injury, but also promote repair after renal IRI (96). As a consequence of renal ischemia, there is an altered localization and expression of complement regulators on tubular cells (97), which makes these cells prone for vigorous complement activation. In addition, apoptotic as well as necrotic cells generated during I/R are potent activators of the complement system (75-78). Therefore, it is thought that also the complement system is an important contributor to renal injury and inflammatory response following IRI.

1.2 THE COMPLEMENT SYSTEM

The complement system, an essential component of the innate immune system, is a major player in host defense against invading pathogens and at the same time is closely involved in the effective clearance of apoptotic and necrotic cells. The complement cascade was first described in the late 1800s (98) and so named to reflect its capacity to enhance antibacterial activity of humoral immunity. It is a complex cascade of approximately thirty plasma and membrane-bound proteins that are stratified according to their respective surface recognition patterns into three major pathways. i.e. the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP). Each pathway has its own characteristics of target recognition, activation and regulation, but all converge at the level of C3,

the central component of the complement system (Fig 6). Once sufficient C3 is activated and deposited, generation of the membrane attack complex (MAC) is initiated, resulting in cytolysis of the target cell (99).

1.2.1 Classical pathway

The CP is activated via binding of C1q to the Fc-tails of immunoglobulins (i.e. IgG and IgM bound to their antigen), acute phase proteins, DNA, and apoptotic cells or necrotic cell debris. When bound to its substrate, a conformational change of C1q results in the activation of its natural serine proteases C1r and C1s, which are associated with the collagen-like tail of C1q. Activated C1s then cleaves C4 into C4b which becomes covalent linked to the target. Subsequently C2 is cleaved which binds to C4b forming the classical (membrane-attached) C3 convertase, the C4b2a complex. This classical C3 convertase activates and cleaves other C3 molecules to C3b and C3a. During cleavage of C3, the internal reactive thioester bond is exposed (100) and covalently links C3b to its target, thereby functioning as an opsonin directing effective clearance by phagocytes via the C3b receptor CR1 (99).

1.2.2 Lectin pathway

The LP is activated in response to binding of the pattern recognition molecule Mannan-binding lectin (MBL) as well as L-ficolin and H-ficolin to various carbohydrate ligands. MBL is the major recognition molecule of the LP of complement activation. Activation of this pathway via MBL and ficolins resembles the CP, but (instead of C1s) the MBL-associated serine proteases (MASP)-1 or -2 are responsible for the activation of C4 and C2 (99). Single-nucleotide polymorphisms in both structural and regulatory parts of the MBL gene have been found to lead to large inter-individual variations in the concentration of functional MBL (0-4000 ng/ml) in plasma (101).

1.2.3 Alternative pathway

The AP is continuously activated at a low level (so-called tickover), does not require C4, and is tightly regulated by complement regulatory proteins, which are lacking on e.g. pathogens. The continuous hydrolysis of C3 leads to formation of C3(H2O). Factor B subsequently binds hydrolyzed C3 in the presence of factor D leading to formation of the alternative C3 convertase C3(H2O)Bb after cleavage and activation of factor B by factor D. This process results in continuous low-level production and deposition of C3b on unprotected surfaces. Deposited C3b binds factor B and subsequent cleavage by factor D results in the formation of a highly active C3 convertase, C3bBb. A very important step in this pathway is the stabilization of C3bBb by properdin which increases the lifetime of this

convertase sevenfold. The deposited C3b generated by the classical and lectin C3 convertases (C4b2a) can in turn be transformed in an alternative C3 convertase by binding of properdin and factor B, and cleavage of factor D, generating additional stable C3 convertases (C3bBbP). In this way the AP is a very potent amplifier of both the CP and LP and is thought to account for approximately 80% of deposited C3b (102-104).

1.2.4 Terminal pathway

After formation of the classical C3 convertase C4b2a or the alternative C3 convertase C3bBb, the final pathway (common to all three pathways) may be initiated. Incorporation of an additional C3b molecule in the C3 convertase leads to the formation of the C5 convertase. After activation and cleavage of C5, binding of C6, C7, C8 and multiple C9 molecules takes place resulting in the formation of the MAC, i.e. the C5b-9 complex, a lipid-soluble pore structure which can cause osmotic lysis of cells.

1.2.5 Complement regulators

Because of its tendency for rapid activation and its ability to amplify its own activation, tight regulation of complement is required. To fully prevent self-depletion and excessive deposition on host cell two types of complement

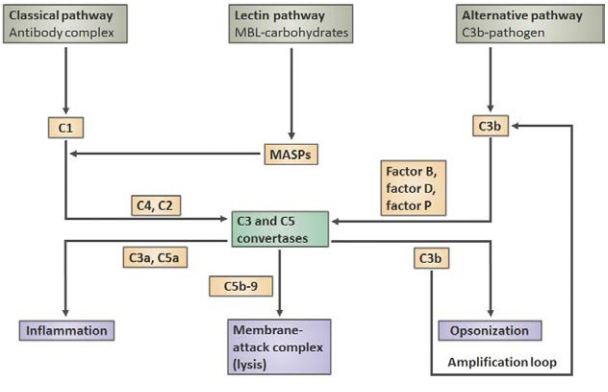

Figure 6. The complement system. The central complement component C3 is activated by three major pathways. The classical pathway is triggered by immune surveillance molecules (such as IgG, IgM and C-reactive protein) that are bound to the activating surface, whereas the lectin pathway is initiated by carbohydrate residues on the activating surface and the alternative pathway is triggered by direct binding of C3b to the activating surface. All three pathways progress to form enzyme complexes (classical or alternative convertases) that cleave either C3 (into C3a and C3b) or C5 (into C5a and C5b). C5b triggers the terminal pathway, which involves the formation of a multimeric membrane attack complex (C5b-9) that creates a pore in the target cell membrane. Specific cell receptors detect the soluble complement effectors (namely, C3a and C5a) and the membrane-bound effectors (namely, C3b and its metabolites inactive C3b (iC3b) and C3d). *Adapted by permission from Macmillan Publishers Ltd: Atkinson et al, Nature Reviews Immunology 7, 9-18 (2007) copyright 2007.*

regulatory proteins (CRPs) are present, i.e. fluid-phase and solid-phase (105). Fluid-phase CRPs circulate in the plasma and include C1 esterase inhibitor (C1INH), C4-binding protein (C4bp), factor H and S protein (vitronectin). C1INH is a serine protease inhibitor belonging to the serpin superfamily, acts as a major inhibitor of complement system activation and prevents uncontrolled activation. C1INH can inhibit both the classical and lectin pathway, and upon complement activation, C1INH binds to activated MASP or C1r and C1s to generate MASP(C1INH) (106) and C1rC1s (C1INH)2 complexes (107-109), which subsequently dissociate from the MBL or C1q molecule, respectively. These complexes are then rapidly cleared from circulation.
C4bp is a multichain inhibitor of the classical and lectin C3 convertase C4b2a and acts as a decay-accelerating factor for C4b2a and as a cofactor for cleavage of C4b to iC4b by the plasma serine-protease factor I.
Factor H is the fluid-phase inhibitor of the alternative C3 convertase C3bBb. In absence of factor H the AP will deplete itself almost completely. Upon binding to C3b, factor H competes with factor B for binding to C3b. Factor H also displaces Bb from the C3bBb convertase (decay-accelerating activity). In addition, factor H acts as a cofactor for factor I in the cleavage of C3b to inactive iC3b. Factor H can bind C3b much more easily in the presence of sialic acid which is present on all host cells but is absent on e.g. pathogens (99).
Every cell in the human body is protected by one or more cell-membrane-anchored complement regulatory protein including CD35 (complement receptor 1; CR1), CD46 (Membrane cofactor protein; MCP), CD55 (Decay accelerating factor; DAF) and CD59 (protectin) which generally prevent or disable the formation of C3b (CD46, CD55) or MAC (CD59). During apoptosis (110), but also following renal ischemia/reperfusion (97), changes in surface molecules occur, leading to loss of CD46 and CD59 allowing complement activation and consequent opsonization by C3b and C4b followed by phagocytosis. Further activation of the complement cascade will lead to generation of C5b-9 and release of C5a inducing an inflammatory environment.

1.2.6 Biological activities of complement

In summary, the main biological activities (99) of the complement system are: (1) the opsonization of pathogens, apoptotic or necrotic cells mediated by the cleavage products of C3 (C3b and iC3b) and C4 (C4b); (2) the recruitment and activation of inflammatory cells by the anaphylatoxins C3a and C5a, which are proteolytically released from C3 and C5 and signal to cells and tissues via two members of the G-protein-coupled receptor family, the C3a receptor (C3aR) and C5a receptor (C5aR) respectively; (3) the direct elimination of pathogens through phagocytosis via complement receptors (e.g.. CR1, Calreticulin/CD91, C1qR) or

by cell lysis as a result of formation of the membrane attack complex (MAC, C5b-9); and (4) the tuning of adaptive immunity by downstream stimulation of B- and T-cells (111).

1.2.7 Complement production

The circulating complement components are mainly produced in the liver and are likely to be present in vast excess of locally generated components. Exceptions to these are properdin and C1q which are mainly produced by neutrophils (112) and DCs (113), respectively. A number of complement components are also produced in the kidney. Ironically, the kidney cells most profoundly targeted by complement during renal I/R, i.e. the PTEC produce vast amounts of complement proteins including C4, C2, C3, factor B and factor H (79). Also, macrophages and dendritic cells present in the interstitium can produce C1q. Pro-inflammatory cytokines upregulate expression of complement components by resident renal cells. In this respect, it was reported that expression of C3 by PTEC was strongly increased during renal allograft rejection in the mouse and in the rat, in association with both ischemic injury and rejection (114;115). In addition to complement production by resident renal cells, also leukocytes infiltrating during inflammation can produce a majority of complement factors (116;117).

1.3 MANNAN-BINDING LECTIN

MBL, initiator of the LP of complement, belongs to a family of proteins called collectins, which consists of a collagenous domain and a carbohydrate recognition domain (CRD) or lectin domain (118-120). The collectins are encoded by a cluster of genes found on the long arm of chromosome 10 in humans and chromosome 14 in mouse. In rodents, the cluster contains SP-A, SP-D, and MBL-A, but there is a second MBL-C gene, thought to have arisen by a gene duplication event, and found on chromosome 19 (121).

1.3.1 MBL structure and ligands

MBL consists of multimers of an identical polypeptide chain of 25 kDa (Fig 7). Each chain comprises four distinct regions: (1) a cysteine-rich N-terminal region; (2) a collagenous domain; (3) a short α-helical coiled-coil domain, the so-called neck region; and (4) a CRD at C-terminal (Fig 7). Three polypeptide chains form a triple helix through the collagenous region, stabilized by hydrophobic interaction and interchain disulphide bonds within the N-terminal cysteine-rich region (122). This trimeric form is the basic structural subunit of the circulating form of MBL. In circulation, this trimeric subunit can form oligomers ranging

from dimers to hexamers. The higher order oligomers of MBL (e.g. tetramers to hexamers) are the effective forms in terms of the protein functions, e.g. the glycan interaction and complement activation on microbial surfaces (123;124). MBL shows selective and calcium-dependent binding to terminal sugars D-mannose, L-fucose and N-acetyl-D-glucosamine (GlcNAc), but not to D-galactose and sialic acid (125;126). All these sugars are commonly found on the surface of many microorganisms. The α-helical coiled-coil domain provides flexibility to the orientation of the CRD of MBL that recognize the specific orientation of hydroxyl groups present in certain sugars such as D-mannose and L-fucose. However, the affinity of a single CRD for one monosaccharide is weak (127), and high-avidity binding therefore requires concurrent binding of multiple CRDs. Patterns of repeating sugar structures on microbial surfaces provide an optimal target for MBL binding. Structural studies have demonstrated that the three sugar binding sites of one MBL subunit (i.e. the triple helix) are separated at a constant distance (45 Å in human; 54 Å in rat) (122;128), offering a flat platform to recognize multiple sugars simultaneously. The clustering of the triple helix (e.g. higher order oligomers) therefore confers the ability to achieve high-avidity binding. In addition to sugar structures, it has been shown that MBL can also bind to phospholipids (129) and nucleic acids (130;131).

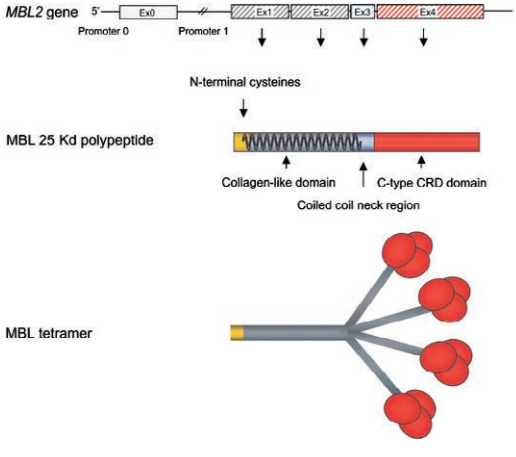

Figure 7. MBL structure. MBL is composed of identical 25 kDa polypeptides, including an N-terminal cysteine-rich region cross linking the polypeptides, a collagen-like stalk region, an α-helic neck region and a C-type carbohydrate recognition domain (CRD). Three polypeptide chains form a triple helix through the collagenous region. This trimeric form is the basic structural subunit of all circulating forms of MBL. MBL consists of oligomers of the subunit, ranging from dimers to hexamers. The affinity of a single CRD for a single carbohydrate is very weak but there is increased avidity of binding when multiple CRDs of MBL interact with carbohydrates, as hexameric MBL has 18 CRDs. The lower panel illustrates one of the predominant forms of MBL found in serum, which consists of four subunits of triple helices of MBL polypeptides. *Adapted by permission from Macmillan Publishers Ltd: Garred et al, Genes Immun. 2006 Mar;7(2):85-94, copyright 2006.*

1.3.2 MBL-associated serine proteases

The effector ability of MBL is facilitated by activation of the LP through a specific interaction between MBL and MBL-associated serine proteases (MASPs). The

minimum functional unit required to activate the LP is a MASP dimer bound to two MBL trimeric units (132). There are three known MASPs that have been termed MASP-1, -2 and -3 (133;134). MASP-1 and MASP-2 are encoded by distinct genes whereas MASP-3 represents an alternative splice form of the MASP1 gene that lacks a serine protease domain. MASP-2 is the functionally most relevant enzyme in initiation of the lectin complement pathway. Ligand binding induces a conformational change in MASP-2 that activates the terminal serine protease domain, which is then able to cleavage C4 and C2, which together forms the classical C3 convertase C4b2a. The roles of MASP-1 and MASP-3 in lectin complement pathway actvation requires further clarification, but recently it was demonstrated that MASP-1 and MASP-3 were able to convert the proenzyme of factor D to an active form, thereby regulating the AP. In addition, MASP-1 was able to activate MASP-2 and MASP-3 as C1r activates C1s (135).

1.3.3 MBL polymorphisms

The main site of production for MBL is the liver. Although it predominantly circulates as a serum protein, MBL has also been detected at various sites, e.g. in middle ear fluid, in synovial fluid of inflamed joint and in nasopharyngeal and vaginal secretion (136;137). In humans, MBL is transcribed from the mbl2 gene, whereas the mbl1 gene is a pseudogene. The mbl2 gene in humans appears to be highly polymorphic (Fig 7). Three mbl2 gene polymorphisms have been identified that are associated with strongly decreased MBL serum concentrations. These single nucleotide polymorphisms (SNPs) (138) are located in codon 54 (B genotype), codon 57 (C genotype), and codon 52 (D genotype) of the first exon, encoding the collagenous tail region of the MBL molecule, and are proposed to hamper the polymerization of the MBL molecule (139-141). Furthermore, SNPs in the promoter of the mbl2 gene modify the basal serum level of MBL (142). A number of studies demonstrated that low serum levels of MBL and MBL gene polymorphisms are associated with decreased pathogen resistance, mainly in childhood but also in adults (141;143-146). An increased susceptibility to infections is predominantly observed in situations in which other defense mechanisms fail, such as in patients with additional immunological defects (147-149), and in patients with other chronic diseases (144;150).

Among the complement deficiencies described in humans, deficiency of MBL has the highest frequency. Depending on ethnicity, the total allele frequency of the B, C and D allele may be above 40 % (151). Since these polymorphisms are not subject to a high negative selection pressure, it has been suggested that the polymorphisms, although conferring LP dysfunction, are also associated with host protection in certain situations (152). In this regard, epidemiological evidence has been provided for a protective role of MBL gene polymorphisms

against the induction of tissue damage in rheumatoid arthritis (153) and against the development of inflammatory bowel disease (154). Furthermore, glomerular deposition of MBL in renal diseases such as post-streptococcal glomerulonephritis (155), IgA nephropathy (137;156-158) and lupus nephritis (159) also supports a potential role for MBL in amplification of tissue injury. In this respect, an unfavorable role for MBL in renal IRI might be possible as well.

1.4 COMPLEMENT IN RENAL I/R

Although not the scope of this thesis, which mainly focusses on the role of complement in renal IRI, it is important to stress that complement activation might not only be involved in the initial phase of kidney transplantation, but may also play a main role in graft rejection following transplantation. Transplantation results in alloantigen-independent and alloantigen-dependent tissue damage. Among the potential alloantigen-independent causes of damage is the condition of the graft before transplantation including type of donor (living, brain-death or cardiac death donor), warm and cold ischemia times, the surgical procedure, and the medical treatment of the recipient, including possible drug toxicity. All these conditions can give rise to tissue injury that can lead to activation of the complement system (160). Accordingly, deposition of complement factors has been observed in graft biopsies obtained early after rejection, both in kidney and heart allografts (161;162). A critical role for local complement production in kidney transplantation has been recently established. Kidneys from C3-deficient mice showed long-term survival when transplanted in MHC-incompatible C3-sufficient recipients without immune suppression, whereas C3-sufficient kidneys from the same strain were subject of rapid acute allograft rejection (115). In this respect, it has been shown that locally produced complement fragments C3a and C5a provide both costimulatory and survival signals to naive CD4+ T cells (163). In addition, antigen-presenting cell-produced C5a and C3a regulates CD4 T-cell help to CD8 T cells, which is required for allograft rejection (164). Together, these experiments provide strong evidence that C3 is a crucial factor in renal allograft rejection.

1.4.1 Complement activation following ischemia/reperfusion

Whether complement activation following ischemia/reperfusion during kidney transplantation is beneficial or detrimental has been intensively studied in several animal models over the past years. Renal I/R generates a massive and dangerous burden of dead cell material, and if not cleared efficiently might lead to inflammation and activation of an early innate response, which may be a

prerequisite for the full development of adaptive alloimmunity and subsequent allograft rejection.

All initiating factors of the complement cascade, including C1q, MBL, ficolins and properdin have been shown to interact with late apoptotic and necrotic cells *in vitro* (75-78) facilitating effective clearance via direct interaction with phagocytic cells or by further opsonzation via activation of the complement cascade. In this respect, opsonization by complement factors followed by efficient clearance could therefore dampen inflammation. However, the role of complement in clearance of dying tubular cells following renal I/R is unclear. Infiltration of professional phagocytic cells, e.g. macrophages in renal IRI is well-known (94;95), however this infiltrate remains mostly interstially and is very rarely observed passing the basolateral membrane entering the lumen of the tubule. Dying tubular cells detach from the basement membrane ending up in the lumen where they obstruct tubular flow. These casts may remain there for several days following AKI. In this respect, a different mechanism for apoptotic and necrotic cell clearance has been proposed recently. Expression of Kidney-injury molecule (KIM)-1 on PTEC, might transform these cells into semi-professional phagocytes (31). KIM-1 is a type-1 membrane receptor that is the most highly upregulated protein in the proximal tubule of the injured kidney and functions as a phosphatidylserine receptor, that recognizes and internalizes apoptotic cells. KIM-1 also functions as a scavenger receptor, mediating the uptake of modified low-density lipoprotein and necrotic cellular debris. The role of complement herein is unknown, however, it is clear that activation of the complement system is one of the hallmarks of renal IRI.

Importantly, several studies mainly performed in mice have shown that complement activation following IRI is harmful and that deletion or inhibition of complement proteins protects against renal IRI, indicating that complement activation following reperfusion might induce further tissue injury and inflammation (165-171). Terminal complement cascade activation including release of C5a and formation of C5b-9 has been shown to be one the factors that contribute to tubular injury. Reperfusion of the kidney following ischemia induces endothelial activation and release of nitrous oxide leading to vasodilatation and leakage of complement components into the interstitial space. Additionally, tubular cells are able to produce complement components locally (79). In combination with a decreased expression of complement regulators at the basolateral side following I/R, viable as well as apoptotic or necrotic tubular cells are easily targeted for (terminal) complement activation. It is therefore not surprising that (terminal) complement activation is observed following renal I/R. Several studies performed in mice deficient for C3, C5, C6 (171) have shown (partial) protection against renal IRI. In addition, a very elegant kinetic IRI study

performed in mice (166) showed that the first C3 deposition was observed several hours after reperfusion and was localized on cellular debris and injured tubular epithelial cells. Intrarenal depositions of C6 were seen at 12 hours after reperfusion and increased over time. C5b-9 deposition was first observed after 18 hours of reperfusion and was distributed similar to C6 deposition, although more intense in tubular casts. Moreover, this study also indicates that inhibition of C5 protects against renal IRI with reduced renal dysfunction and neutrophil influx into the kidney. Interestingly, inhibition of C5 totally prevented C5b-9 formation, but also reduced C3 deposition, clearly indicating that terminal complement activation results in additional local inflammation and collateral damage leading to more tissue destruction and renal dysfunction in rodents. In rats, kinetics of complement activation is more delayed with first signs of complement activation and deposition of C3 at 24 hours of reperfusion (Fig 8). In human thus far, in-depth studies for the role and kinetics of complement activation in renal IRI are still lacking.

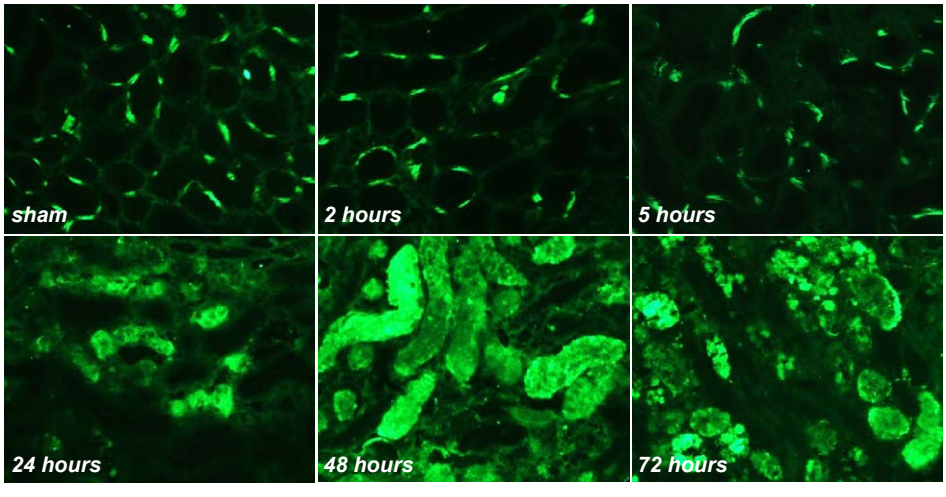

Figure 8. Production and deposition of complement component C3 after renal I/R. Normal rat kidney (sham) showing characteristic half-moon shaped staining of C3, reflecting local renal production. Upon renal ischemia (45 min clamping), first signs of complement activation and deposition of C3 on tubular cells are observed after 24 hours of reperfusion peaking at 48 hours. At 72 hours of reperfusion, deposition of C3 is still present on desquamated tubular epithelial cells and tubular casts in the lumen of the tubules.

1.4.2 Pathogenic role of complement activation

Although local complement activation might be injurious in renal IRI, the question remains whether activation of the complement cascade by tubular cells is the very initial trigger for cellular injury following reperfusion or that

complement merely aggravates inflammation and local tissue injury by activation of the terminal complement cascade on injured and dead cells. Currently, only one study has been published that supports the hypothesis that terminal complement activation, i.e the lytic C5b-9 complex is the initial trigger for tubular cell injury following reperfusion (171). In this study, a central role for the terminal complement cascade in renal IRI was suggested from C6-deficient mice which were partially protected. Only a minor role was ascribed to the release of C5a and subsequent recruitment of neutrophils into the kidney, given that treatment with an antibody to C5 did not show any additional protection in C6-deficient mice. In contrast, a study performed in rats (172) demonstrated that blocking of the C5aR pathway by a specific C5a receptor antagonist had a protective effect against renal dysfunction following I/R. In another study (166), blocking of C5 cleavage in mice using a monoclonal antibody and thereby preventing C5b-9 formation only abrogated late I/R-induced apoptosis and inflammation, whereas early apoptosis was not prevented, indicating that C5a does not have a direct harmful effect on tubular cells. Since both C5a and C5b-9 have been reported to be involved in the induction of inflammatory cytokines and chemokines, such as TNF-alpha, KC, and MIP-2 (173-178), this might explain the observed protective effects. Given that tubular cell death early following reperfusion is not prevented by blocking C5, this suggests that other cell death-inducing mechanisms independent of (terminal) complement activation might be involved.

1.4.3 Differential pathway activation between species

Although the role of complement in the early pathogenesis of renal IRI has not been completely elucidated, it is clear that complement activation induces additional local inflammation and collateral damage leading to more tissue destruction and renal dysfunction. Therefore, therapeutic interference with complement activation might be an interesting option to treat and ameliorate renal IRI. However, to therapeutically target complement in renal IRI, it is important to delineate which pathways of complement activation are involved since blocking of all complement pathways early after transplantation could lead to a higher risk of e.g. urinary tract infections, a major complication following kidney transplantation.

Complement activation in the mouse kidney is mainly attributed to the alternative pathway (AP) of complement activation (179;180). Renal IRI in mice does not induce antibody deposition and subsequent CP activation (181). Furthermore, mice lacking a functional AP are protected against renal IRI (179). In contrast, RAG-1-/- mice incapable of producing antibodies are not protected, suggesting that renal IRI in mice is not mediated via the CP (181). These data have been

confirmed in C3-/-, C5-/- and C6-/- mice which are protected, whereas C4-/- mice are not (181;182). This seems to be in contrast to murine models of IRI in heart, skeletal muscle, intestine and limb, which are all dependent on natural IgM and CP or LP activation.

Intriguingly, although C4-deficient mice were not protected, studies using MBL-knockout mice have shown a protective effect of MBL deficiency in the setting of renal IRI (168), and also renal deposition of MBL has been demonstrated. In line with this, it has been demonstrated in several mouse models of IRI, that MBL in association with MASP-2 can bypass C4 and directly cleave C3 (183) followed by further amplification via the AP. This might explain why MBL-deficient mice are protected against renal IRI, although other effectors functions of MBL might be involved. Glycosylated tubular meprins, which bind MBL (167), might be involved in the activation of complement in the mouse kidney.

In contrast to these studies in mice, it was recently shown in a porcine IRI model that both the CP and LP might be involved. Reduced IRI is observed when pigs are treated with human C1 Inhibitor (C1INH), an inhibitor of both the CP and LP. An important difference between these species is the presence of peritubular C4d staining in pigs following reperfusion (165), which is completely absent in mice. This suggests that classical and lectin pathway activation by C1q and MBL leading to C4 deposition in the kidney following reperfusion in pigs is occurring, but is virtually absent in rodents. Importantly, C1INH has several other effector functions besides regulating complement, including regulation of coagulation and vascular permeability and inhibition of apoptosis (184-187). These effector functions might therefore also explain the observed protective effect, which lead to reduced renal IRI and subsequent less C4 and C3 deposition on tubular cells. The role and pathways of complement activation in porcine, but also human IRI is therefore not completely been elucidated yet.

Interestingly, two studies by Berger et al (101;188) were showing that high serum levels of MBL in human are associated with inferior renal allograft survival following clinical kidney transplantation, suggesting an unfavorable role for MBL in kidney transplantation. However, we can only speculate whether this was due to involvement of MBL in the initial phase during transplantation or in later rejection episodes. Altogether, these findings point towards important species-specific differences in complement activation following I/R, however in-depth studies on the mechanism and involvement of different pathways of complement activation in humans are still lacking. The possible differences in the mechanisms of complement activation by mouse and human tubular cells might have important implications for the interpretation of experimental data obtained in mice. To successfully develop therapeutic interventions targeted towards different pathways of complement activation, it is essential to establish

the validity of murine data relative to what takes place in the human situation. Therefore, there is a great demand for studies analyzing the activation of complement pathways in human kidney transplantation.

1.5 THESIS AIM AND OUTLINE

The aim of the research described in this thesis was to study the role of complement in renal ischemia/reperfusion injury (IRI) and to delineate the contribution of the different complement pathways involved. So far, in human renal IRI, the activation pathways of complement by ischemic proximal tubular epithelial cells (PTEC) are still incompletely elucidated. In **chapter 2** we therefore established an *in vitro* model to simulate IRI on human and mouse PTEC by culturing these cells under normoxic or hypoxic conditions and then investigated the subsequent effects on complement activation following reoxygenation (reperfusion). We specifically focused on the question which pathway(s) of complement activation are initiated by human and mouse PTEC after hypoxic stress. In **chapter 3** we addressed the lack of evidence for involvement of complement in human IRI. We assessed the formation and release of C5b-9 during early reperfusion in clinical kidney transplantation in living donor, brain-dead donor, and cardiac dead donor kidney transplantation. Complement activation following I/R may take place in both tubular and vascular compartments. Therefore, we systematically measured terminal complement activation during early reperfusion in human kidney transplantation in both the tubular compartment by immunohistochemistry and the intravascular compartment by selective arteriovenous measurements over the transplanted kidney. In **chapter 4** we studied whether C5b-9 could also be detected in urines of transplant recipients early after transplantation. In addition, we investigated the possibility whether in proteinuric urine, which is common following transplantation, C5b-9 might be generated independent of a renal contribution.

Based on previous clinical studies that high serum levels of MBL were associated with inferior renal allograft survival following clinical transplantation, we examined in **chapter 5** the role of MBL in the pathophysiology of renal IRI and explored the therapeutic targeting of MBL in a rat model of renal IRI. We identified an entirely novel role for MBL in mediating reperfusion-induced kidney injury following ischemia which is completely independent of complement activation. In **chapter 6** we studied the mechanism by which MBL might mediate tubular injury following renal I/R. Vascular leakage results in exposure of tubular cells to MBL, which was shown to be the primary culprit of tubular injury. Recombinant human C1 inhibitor (rhC1INH) is a serine protease inhibitor that inhibits

complement activation, reduces vascular permeability and interacts with MBL. In **chapter 7** we therefore explored the therapeutic application of rhC1INH in renal IRI and studied whether rhC1INH is able to attenuated MBL-mediated kidney injury. In **chapter 8** we investigated the impact of short- and long-term IRI on vascular integrity, pericytes and angiopoietin expression. Finally, in **chapter 9** the findings presented in this thesis are critically discussed and the possible implications for kidney transplantation are presented.

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