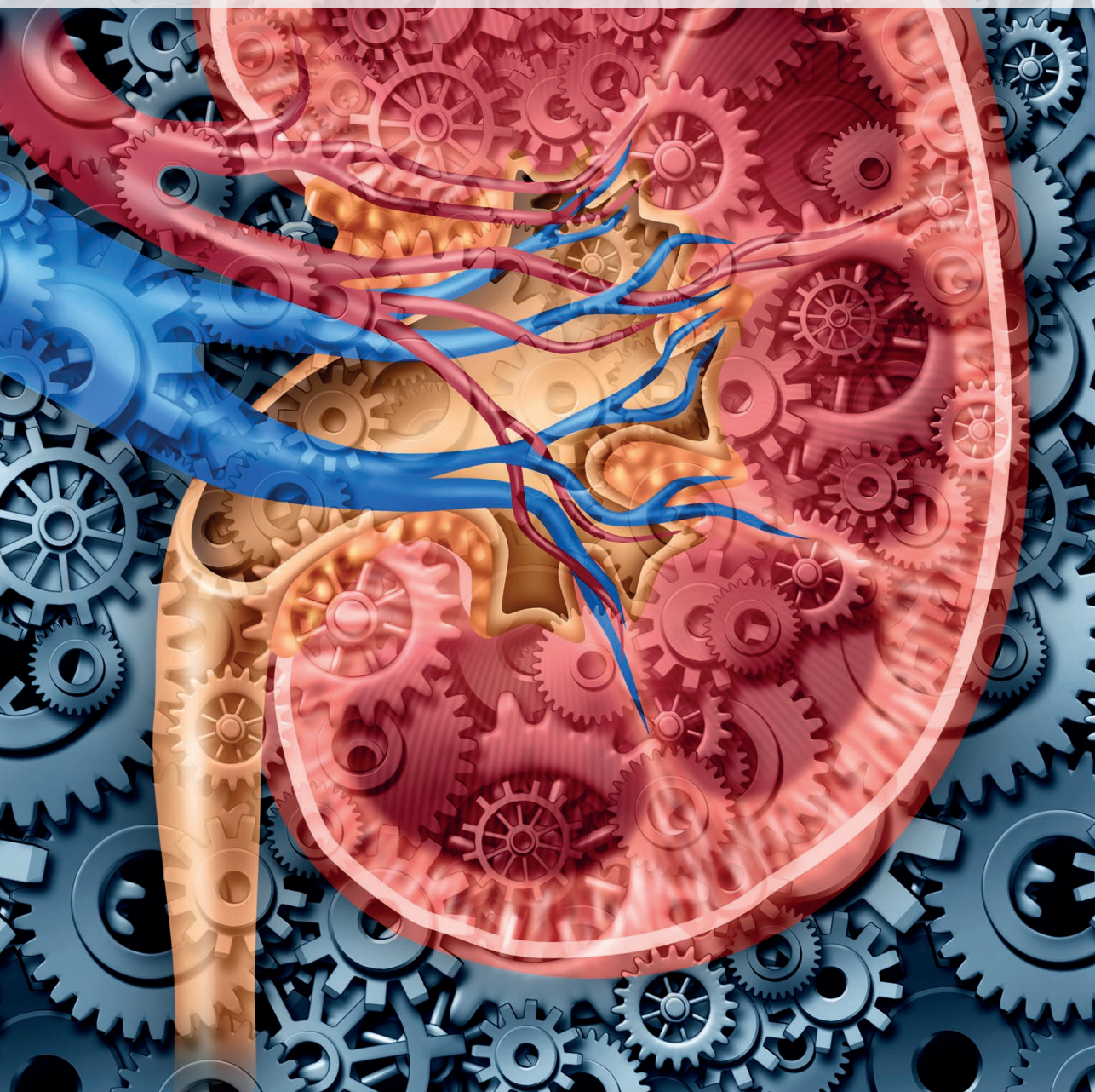
Pathogenic role of complement in renal ischemia/reperfusion injury

Pieter van der Pol



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Voor mijn ouders en Karin

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CHAPTER 1

General introduction

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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.

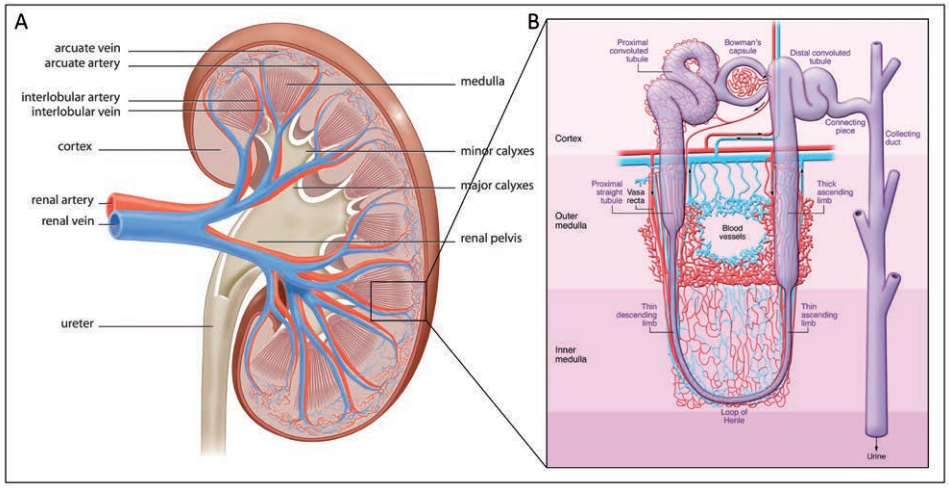

Figure 1. Normal kidney and nephron with medullary microvascular anatomy. Anatomy of the kidney (A) and a nephron (B) with regions identified. Outer medulla vasculature is shown with capillaries in red and venous system in blue. The vasa recta with countercurrent exchange of oxygen resulting in a gradient 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,

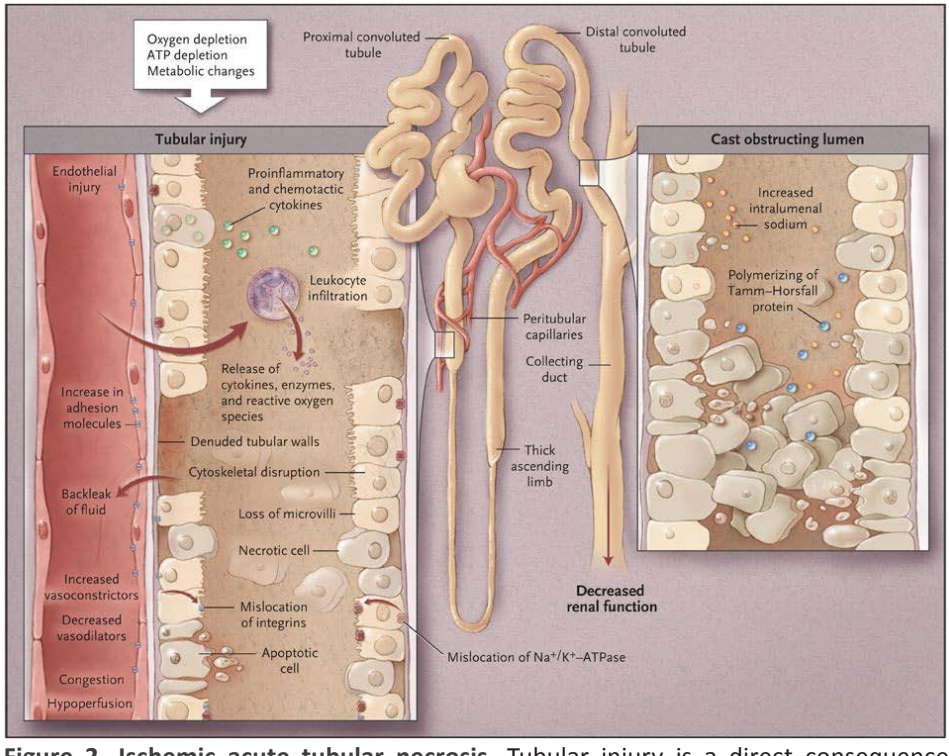

Figure 2. Ischemic acute tubular necrosis. Tubular injury is a direct consequence of metabolic pathways induced by ischemia but is potentiated by inflammation and microvascular compromise. Acute tubular necrosis is characterized by shedding of epithelial cells and denudation of the basement membrane 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).

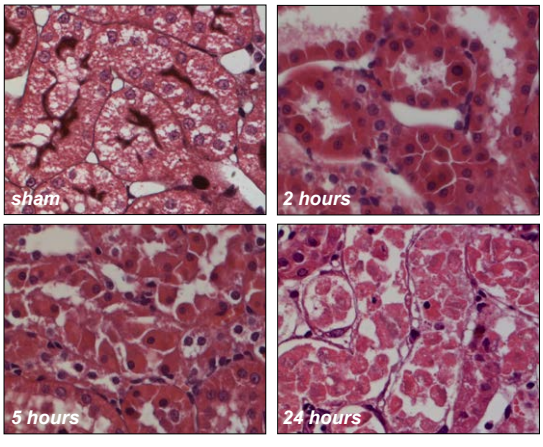

Figure 3. Ischemic acute tubular necrosis in the outer medullary region following I/R in rats. ATN is characterized by loss of the brush border and tight and adherens junctions between tubular epithelial cells (2h after I/R) followed by detachment from the basement membrane (5h after I/R). Basement membranes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

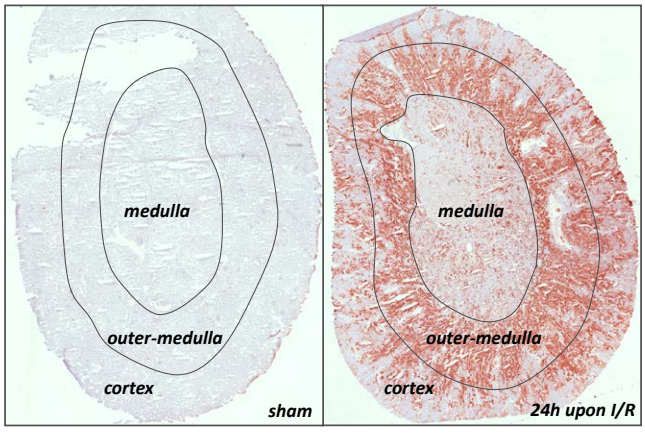

Figure 4. Kidney injury molecule (KIM)-1 after I/R. Whole rat kidney staining of KIM-1 in an untreated (sham) or clamped (45 min) kidney 24 hours after reperfusion. KIM-1 is most present in the outer medulla, which harbors the proximal tubular epithelial cells in the S3 segment of the nephron.

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.

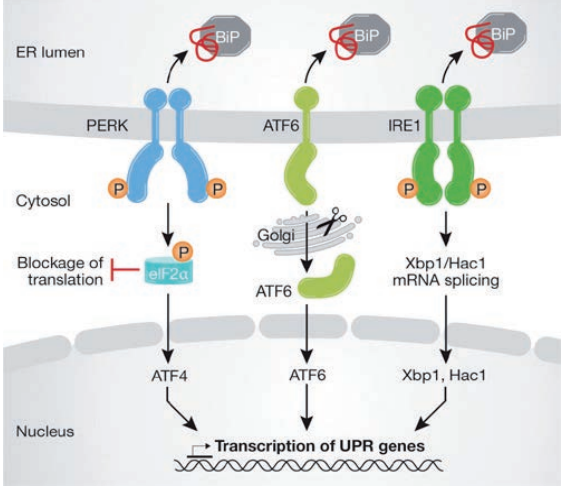

Figure 5. Stress sensors in the ER-stress response. Accumulation of mis- or unfolded proteins in the ER-lumen results in ER-stress and activation of the ER-stress or unfolded protein response (UPR). Release and binding of GRP78/BiP to mis- or unfolded proteins activate the ER-stress sensors PERK, ATF6 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

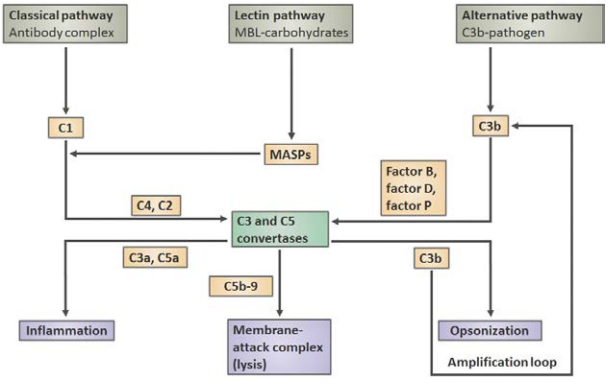

Figure 6. The complement system. The central complement component C3 is activated by three major pathways. The classical pathway is triggered by immune surveillance molecules (such as IgG, IgM and C-reactive protein) that are bound to the activating surface, whereas the lectin pathway is initiated by 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).

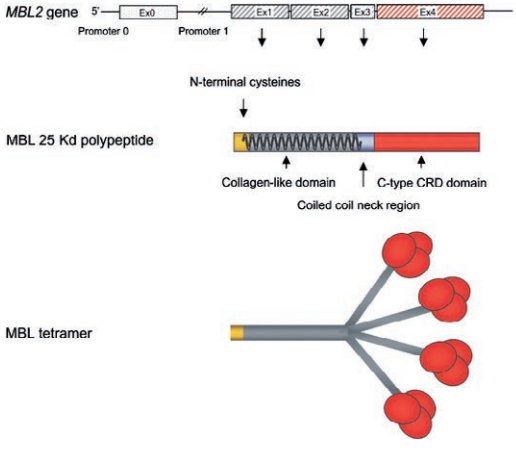

Figure 7. MBL structure. MBL is composed of identical 25 kDa polypeptides, including an N-terminal cysteine-rich region cross linking the polypeptides, a collagen-like stalk region, an α-helic neck region and a C-type carbohydrate recognition domain (CRD). Three polypeptide chains form a triple helix 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.

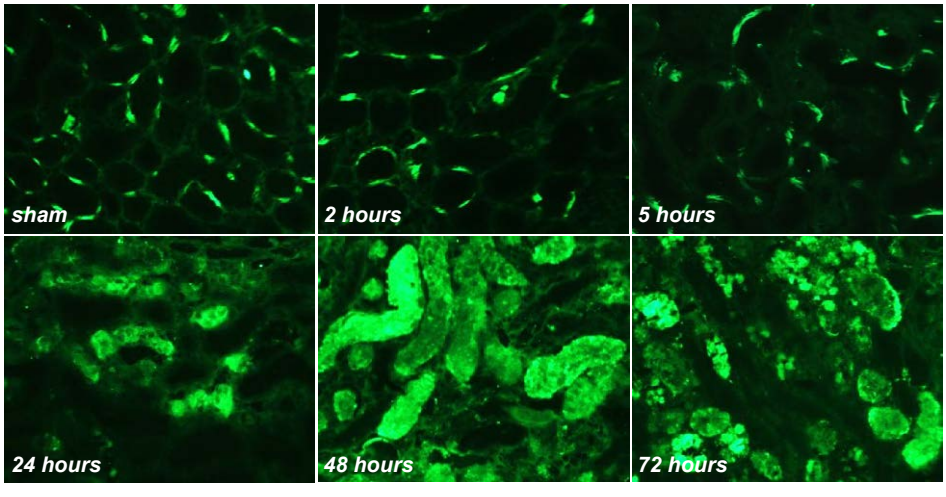

Figure 8. Production and deposition of complement component C3 after renal I/R. Normal rat kidney (sham) showing characteristic half-moon shaped staining of C3, reflecting local renal production. Upon renal ischemia (45 min clamping), first signs of complement activation and deposition of C3 on tubular 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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CHAPTER 2

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Natural IgM antibodies are involved in the activation of complement by hypoxic human tubular cells

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ABSTRACT

Ischemia/reperfusion injury (IRI) has a major impact on graft survival after transplantation. Renal proximal tubular epithelial cells (PTEC) located at the cortico-medullary zone are relatively susceptible to IRI and have been identified as one of the main targets of complement activation. Studies in mice have shown an important role for the alternative pathway of complement activation in renal IRI. However, it is unclear whether experimental data obtained in mice can be extrapolated to humans. Therefore, we developed an *in vitro* model to induce hypoxia/reoxygenation in human and mouse PTEC and studied the role of the different pathways of complement activation. Exposure of human PTEC to hypoxia followed by reoxygenation in human serum resulted in extensive complement activation. Inhibition studies using different complement inhibitors revealed no involvement of the alternative or lectin pathway of complement activation by hypoxic human PTEC. In contrast, complement activation by hypoxic murine PTEC was shown to be exclusively dependent on the alternative pathway. Hypoxic human PTEC induced classical pathway activation, supported by studies in C1q-depleted serum and the use of blocking antibodies to C1q. The activation of the classical pathway was mediated by IgM through interaction with modified phosphomonoesters exposed on hypoxic PTEC. Studies with different human sera showed a strong correlation between IgM binding to hypoxic human PTEC and the degree of complement activation. These results demonstrate important species-specific differences in complement activation by hypoxic PTEC and provide clues for directed complement inhibition strategies in the treatment and prevention of IRI in the human kidney.

INTRODUCTION

Ischemia/reperfusion injury (IRI) occurs upon reperfusion of vascularized tissue after an extended period of ischemia. It is an inevitable event in organ transplantation. Clinical and experimental studies have shown that renal IRI has a major impact on short- and long-term graft survival after organ transplantation and accounts for delayed graft recovery and associated morbidity and mortality (1;2). Restoration of blood flow to ischemic tissue initiates a cascade of inflammatory events including endothelial dysfunction, neutrophil sequestration and complement activation (C-activation) which all contribute to post ischemic injury (3).

The complement system is a complex cascade of proteins that can be activated by three different pathways (4). Each pathway is activated by a different set

of pattern recognition molecules. The classical pathway (CP) is initiated by direct binding of C1q to e.g. apoptotic cells, or by binding to antigen-antibody complexes. The lectin pathway of complement (LP) is activated by interaction of Mannan-binding lectin (MBL) or ficolins with sugar moieties. CP and LP activation both lead to deposition of C4 and C2 which result in the generation of the classical C3 convertase that is able to cleave C3. The alternative pathway (AP) is continuously activated at a low level (so-called tickover), does not require C4 and is tightly regulated by membrane-bound and soluble complement regulators. Furthermore, properdin can act as a focal point of AP mediated C-activation upon binding to its ligand (5). All pathways converge at the level of C3 and further downstream activation leads to formation of the membrane attack complex C5b-9 (MAC) (4).

Complement activation is a key feature of renal IRI, as has been demonstrated both in the clinical setting as well as in experimental models (6). Moreover, interference with C-activation reduces IRI. Studies in mice have suggested that the AP is predominantly activated in IRI. Mice deficient in factor B (7), an crucial constituent of the AP, or mice treated with anti-factor B antibodies (8) show reduced injury, whereas C4-deficient mice were shown to be as susceptible to renal IRI as wildtype mice (9). Nevertheless, other pathways of C-activation have also been implicated. Deposition of MBL was observed in mouse kidneys after renal IRI (10) and also deficiency of MBL partially protects mice against renal IRI (11). In pigs, different components of the CP and LP were detected after renal ischemia and therapeutic intervention with C1INH, which interferes with both the LP and CP, was successful (12).

In the heart, muscle and the intestine, C-activation after ischemia depends on naturally occurring IgM antibodies to intracellular antigens which are externalized upon ischemia (13-15). The role of these antibodies in renal IRI is controversial (16;17).

Both endothelial and epithelial cells in the kidney seem to be targets for C-activation following IRI. In the kidney, several studies have implicated the cortico-medullary proximal tubular epithelial cell (PTEC) as an important target (9;18). Renal IRI is associated with a reduction in membrane-bound complement regulators on PTEC (18). Moreover PTEC interact with properdin (19), which can serve as a focus for AP activation (5).

So far, in human renal IRI the activation pathways by PTEC are still incompletely elucidated. In the present study, we developed an *in vitro* model to induce hypoxia/reoxygenation and investigated the subsequent effects on C-activation by hypoxic human and mouse PTEC. We specifically focused on the question which pathways of C-activation are initiated by the PTEC after hypoxic stress. We demonstrate that C-activation by human PTEC as a result of hypoxia/

reoxygenation primarily occurs via the CP of complement, and is dependent on both IgM antibodies and C1q. In contrast, hypoxia-induced C-activation by mouse PTEC primarily occurs via the AP.

METHODS

Cell culture

Immortalized human renal proximal tubular epithelial cells (HK-2, kindly provided by M. Ryan, University College Dublin, Ireland)(20) were grown in serum-free DMEM/HAM-F12 (Bio-Whittaker, Walkersville, US) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen, Breda, the Netherlands), insulin (5 µg/ml), transferrin (5 µg/ml), selenium (5 ng/ml), tri-iodothyronine (40 ng/ml), epidermal growth factor (10 ng/ml) and hydrocortisone (36 ng/ml, all purchased from Sigma, Zwijndrecht, the Netherlands). Primary human PTEC were isolated from pre-transplant biopsies or from kidneys not suitable for transplantation and cultured as described earlier (21).

Immortalized mouse renal proximal tubular epithelial cells (IM-PTEC, kindly provided by Dr. G. Stokman, Gorlaeus Laboratories Leiden, Netherlands) were derived from a single proximal tubular epithelial cell of an Immorto mouse (Charles River, Maastricht, The Netherlands) based on the double expression of aquaporin-4 and CD10/neprilysin. Immorto mice express a temperature sensitive, interferon gamma dependent variant of the SV40 large T antigen. Cells are grown under permissive conditions at 33°C in DMEM/HAM-F12 (Bio-Whittaker,) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen), insulin (5 µg/ml), transferrin (5 µg/ml), selenium (5 ng/ml), tri-iodothyronine (40 ng/ml), prostaglandin E1 (5 ng/ml), hydrocortisone (36 ng/ml, all purchased from Sigma), mouse IFN-gamma (10 ng/ml; R&D systems, Uithoorn, The Netherlands) and maintained for at least one week under restrictive conditions at 37°C in the absence of IFN-gamma to ensure re-differentiation.

Induction of hypoxia/reoxygenation and necrosis

To simulate an ischemic event, cells were grown to confluence in 24-well culture plates at 37 °C and subjected to normoxia (21% O_2 and 5% CO_2) or hypoxia (5% $C0_2$ and 95% N_2) for 48 hours in a humidified modular incubator chamber (Billups-Rothenberg, Del Mar, US). Since it is known that cultured PTEC are resistant to hypoxia in high glucose levels (22), culture medium of the HK-2 cells, primary cultured human PTEC and mouse IM-PTEC was replaced by identical culture medium, but only containing 2 mM glucose, before induction of hypoxia.

To simulate the reperfusion phase, cells were washed and reoxygenated in 5% or 30% pooled human serum (NHS) or mouse serum (NMS) respectively, diluted in DMEM/HAM-F12 for 1h at 37 °C. As a control, cells were reoxygenated in medium without serum. Finally cells were washed in PBS, harvested with non-enzymatic dissociation buffer (Sigma) and resuspended in FACS-buffer (1% BSA, 0.02 % sodium azide and 2,5 mM CaCl2 in PBS). Necrosis was induced by incubating fresh HK-2 cells and IM-PTEC at 56°C for 30 minutes, after which the cells were incubated with 5% NHS or 5% NMS for 1h at 37 °C.

Analysis of complement activation pathways

In order to elucidate which pathways of complement are involved in C-activation by hypoxic cells, several complement inhibitors were evaluated. EDTA, at a final concentration of 5 mM, was used to inhibit all pathways of C-activation. MgEGTA, at 5 mM, was used to block both the CP and LP. To further assess the role of the LP, D-Mannose (200 mM; Sigma) or increasing doses of mAb 3E7 (anti-MBL mAb kindly provided by Dr. T. Fujita, Fukushima, Japan), which both inhibit the binding of MBL to its ligands, were used. To assess the role of the CP, increasing doses of mAb85 (mAb anti-human C1q, kindly provided by Prof. C. E. Hack, Sanquin, Amsterdam, the Netherlands) were used. MAb85 is directed against the globular head regions of C1q and inhibits binding of C1q to activators such as IgM and aggregated IgG (23). To further study CP activation the binding of IgM (5-100 µg/ml) to normoxic and hypoxic HK-2 cells was assessed. Functional consequences of IgM binding to hypoxic HK-2 cells were studied using 5% or 30% C1q- or IgM-depleted human serum. As a control the serum was reconstituted with purified C1q (50 µg/ml) or IgM (100 µg/ml) respectively. IgM-dependent C-activation by mouse PTEC was studied by incubating hypoxic mouse IM-PTEC with 5% serum derived from immunoglobulin-deficient RAG -/- mice. Furthermore, the role of IgM in C-activation was studied using the phosphatemonoester phosphorylcholine (Sigma), an antigen for natural IgM. Phosphorylcholine (20mM) was first preincubated with 5% NHS for 15 min at room temperature and next incubated with hypoxic HK-2 cells for 1h at 37 °C. Binding of serum IgM and deposition of C3, C4 and C5-b9 on normoxic and hypoxic human PTEC was studied using 5% NHS from eleven healthy donors diluted in serum-free DMEM/HAM-F12 culture medium and incubated on the cells for 1 h at 37 °C.

FACS analysis

After incubation with purified IgM or serum as a source of complement, cells were washed in PBS, harvested with non-enzymatic dissociation buffer (Sigma) and resuspended in FACS-buffer (1% BSA, 0.02 % sodium azide and 2,5 mM CaCl2 in PBS). Depositions of C3, C4, C5b-9 and binding of IgM on human

PTEC were detected using mouse monoclonal antibodies against human C3 (RFK22, Laboratory of Nephrology, Leiden, the Netherlands), human C4 (C4-4A, anti-C4, kindly provided by Prof. C. E. Hack,), human C3d (Quidel, San Diego, USA), human C4d (Quidel), human C5b-9 (mAb AE11, kindly provided by Dr T. E. Mollnes, Nordland Central Hospital, Bodo, Norway) and human IgM (HB57, hybridoma obtained from the American Type Culture Collection, Manassas, VA) respectively, followed by RPE-conjugated polyclonal goat anti-mouse Ig (DAKO, Glostrup, Denmark,). Deposition of C3 on mouse PTEC was detected using a rabbit polyclonal antibody anti-mouse C3 (in house generated) (24), followed by RPE-conjugated polyclonal goat anti-rabbit Ig (DAKO). All antibody incubations were performed on ice for 30 min. Cell surface staining was assessed using a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA). Propidium iodide (PI, 1µg/ml, Molecular Probes, Leiden, the Netherlands) and Annexin V-FITC (25 µg/ml, VPS Diagnostics, Hoeven, the Netherlands) was used for exclusion of apoptotic and necrotic cells.

Assessment of functional lectin pathway activity by ELISA

Functional activity of the LP was assessed using mannan-coated plates as previously described (25). Shortly, NHS preincubated with L- or D-Mannose (Sigma) was incubated for 1 hour at 37°C to allow C4 and C3 deposition in the well. The plates were washed and incubated with dig-conjugated C4-4A (anti-human C4) or RFK22 (anti-human C3), followed by HRP-conjugated F(ab')2 from goat IgG anti-dig (Boehringer Mannheim, Mannheim, Germany). After washing, C3 deposition was quantified with ABTS (Sigma). The OD at 415 nm was measured using a microtiter plate reader.

Complement and serum reagents

All samples were collected and experiments were performed according to the guidelines of the ethics committee of the Leiden University Medical Center. As a source of complement, pooled normal human serum (NHS) from healthy donors and pooled mouse serum from C57BL/6 (NMS) and RAG-/- mice was divided into aliquots and stored at -80°C until used. Human C1q-depleted serum was generated as previously described(25). The C1q-depleted serum showed normal LP and AP activity in hemolytic assays and could be completely restored with purified C1q. Human IgM-depleted serum was generated by immune adsorption using Biogel-coupled anti-human IgM monoclonal antibodies (HB57) at a high salt concentration to prevent C-activation during the procedure and showed normal activity in all three complement pathways. Human C1q (26) and IgM (25) were purified as previously described.

Statistical analysis

Correlation analysis between variables was performed by linear regression and the significance of differences between groups was calculated by Student's t-test using GraphPad Prism software (GraphPad Software, San Diego, CA). Differences with $P<0.05$ were considered significant.

RESULTS

Complement activation by hypoxic HK-2 cells and primary human PTEC

In order to establish an *in vitro* model to simulate IRI on PTEC we cultured HK-2 cells under normoxic or hypoxic conditions. Forty-eight hours of hypoxia were required to induce hypoxic stress which was accompanied by morphological changes with rounding of cells and loss of tight junctions (Fig. 1A). Despite these changes, the vast majority of both hypoxic and normoxic cell populations (respectively >75% and > 90%) were still viable at this time point, as determined

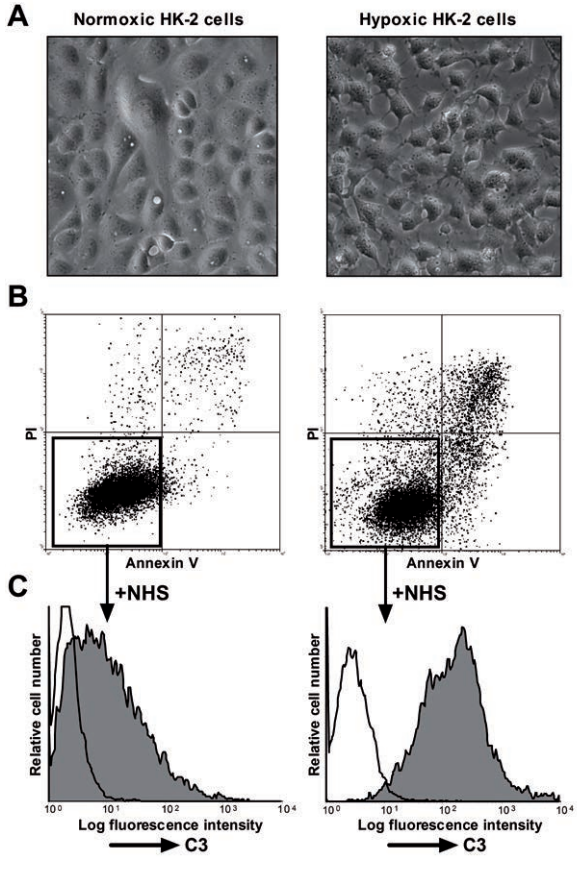

Figure 1. Induction of hypoxic stress. HK-2 cells were subjected to normoxic or hypoxic conditions for 48 hours. Photographs (20x magnification) showing normoxic (a; left) and hypoxic cells HK-2 cells which are stressed, rounded and have lost their tight junctions with neighboring cells (a; right). Cells were stained with PI and Annexin V and apoptotic/ necrotic cells were excluded from further analyses (b). C3 deposition was determined by FACS after 1 hour reoxygenation of cells in 30% NHS (c).

with Propidium iodide and Annexin-V staining (Fig. 1B). In all cases, apoptotic and necrotic cells were excluded from the analysis.
Reoxygenation of the cells in 30% normal human serum (NHS) resulted in extensive C-activation by hypoxic HK-2 cells (Fig. 1C). In line with our previous findings, a low level of C-activation was also observed on normoxic cells (19).
C-activation by hypoxic HK-2 cells was not restricted to deposition of C3 only (Fig. 2B), but was accompanied by deposition of C4 (Fig. 2A) up to the level of C5b-9 (Fig. 2C). Moreover, using monoclonal antibodies to neoepitopes on C4d and C3d, we demonstrated that the detected C3 and C4 on the cell surface reflects activated C-fragments (Fig. 2D, E). Furthermore, in all cases, C-activation could be completely blocked using EDTA, showing that the deposition of C4, C3 and C5b-9 was the result of activation of the complement system (Fig. 2A-E).

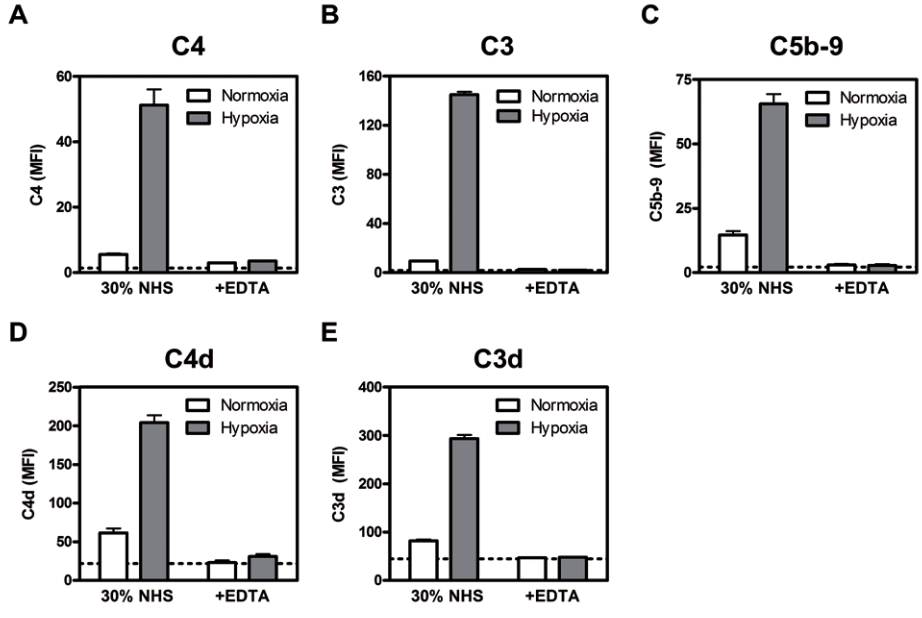

Figure 2. Complement activation by hypoxic HK-2 cells. Normoxic (white bar) and hypoxic (grey bar) HK-2 cells were reoxygenated in 30% NHS and stained for C4 (a), C3 (b), C5b-9 (c), C4d (d) and C3d (e) deposition. As a control complement activation was blocked using EDTA. Dotted lines represent background staining. Results given are the mean (+SD) MFI of triplicate cultures and are representative of 3 independent experiments.

In addition to the HK-2 cell line, also primary human PTEC derived from different donors (n=5) were subjected to 48 hours of hypoxia followed by 1 hour reoxygenation in 5% serum (Fig. 3A, B). While normoxic PTEC induced low-grade C-activation, hypoxia/reoxygenation of these cells resulted in a significant

increase of C-activation and deposition of C3. Interestingly, there was some variance in the degree of C-activation between the five different PTEC (Fig. 3A), indicating that some PTEC were more prone to activate complement after hypoxia/reoxygenation than others. Titration of serum showed that similar complement activation was observed when comparing 5 or 30% serum as a source of complement (data not shown), therefore 5% serum was used for further experiments.

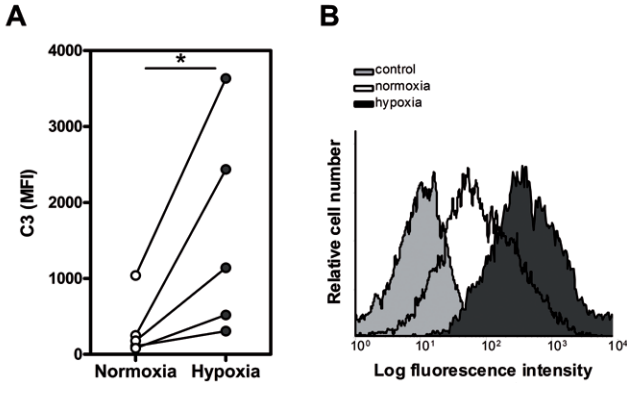

Figure 3. Complement activation by hypoxic primary PTEC. Primary human PTEC (n=5) were subjected to normoxic or hypoxic conditions for 48 hours and reoxygenated in 5% serum for 1 hour. C3 deposition was determined by flow cytometry (a,b). White dots represent normoxic and dark grey dots hypoxic conditions.

A prominent role for alternative pathway activation by hypoxic mouse, but not human PTEC

Although the activation of complement and deposition of C4 on human PTEC (Fig. 2A) already suggested involvement of the CP or LP, most mouse studies of renal IRI have implicated a dominant role for the AP (7;8). Therefore, similar as with the human PTEC, mouse PTEC were cultured under hypoxic conditions for 48 hours and reoxygenated in normal mouse serum (NMS) in the presence or absence of MgEGTA, which blocks both the CP and LP but leaves the AP intact. Indeed, C3 deposition on hypoxic mouse PTEC was not affected by the addition of MgEGTA, indicating that the AP is the predominant pathway activated by hypoxic mouse PTEC (Fig. 4A). In contrast, incubation of hypoxic human HK-2 or hypoxic primary human PTEC with 5% serum in the presence of MgEGTA, almost completely prevented deposition of C3 (Fig. 4A), indicating a major involvement of the CP or LP.

Necrotic human cells are known to bind different complement components, leading to activation of the complement system. To study whether C-activation by necrotic human and mouse PTEC also follow different pathways of C-activation, these cells were rendered necrotic and incubated with human or mouse serum respectively in the presence or absence of MgEGTA. Indeed, mouse PTEC again exclusively activate the AP as MgEGTA did not have any inhibitory effect (Fig. 4B). Under these conditions human necrotic PTEC now also show some activation

of the AP. These data show that hypoxic and necrotic mouse PTEC exclusively activate the AP, while hypoxic human PTEC primarily activate the CP/LP and necrotic human PTEC activate both the AP and CP/LP.

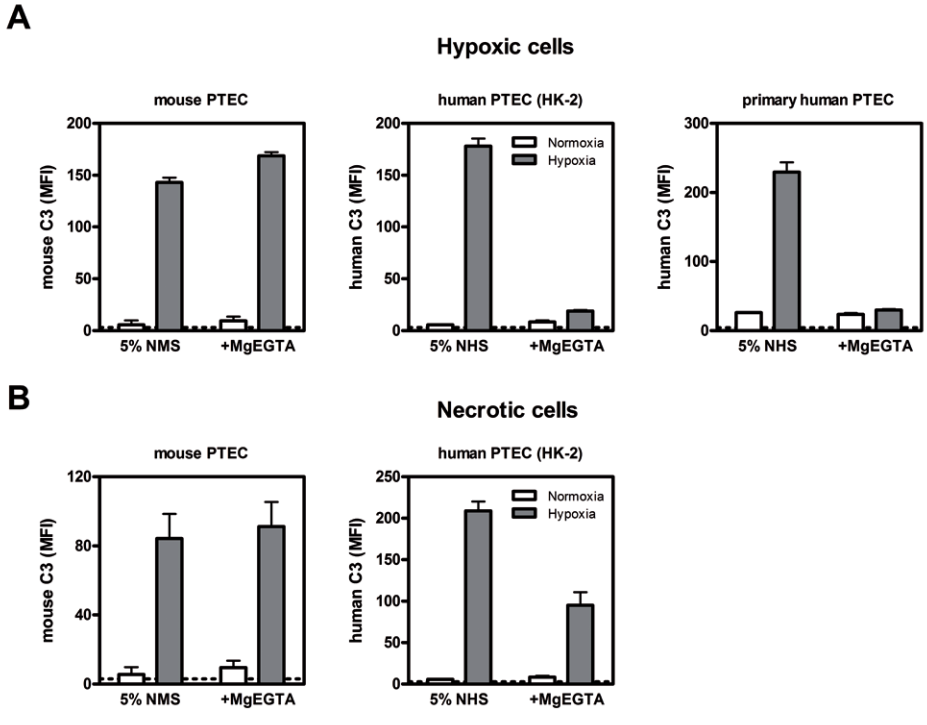

Figure 4. In contrast to mouse, hypoxic human PTEC hardly activate the alternative pathway. (a) To study the contribution of the alternative pathway human (HK-2) and mouse PTEC (IM-PTEC) were rendered hypoxic (a) or necrotic (b) and incubated with 5% NHS or NMS respectively, in the presence or absence of MgEGTA, to block classical and lectin pathway activation. C3 deposition was determined using flow cytometry. White bars represent normoxic and grey bars hypoxic conditions. Results given are the mean (+ SD) MFI of triplicate cultures and are representative of 3 independent experiments.

No contribution of lectin pathway activation by hypoxic human PTEC

Several studies in mice and humans have shown a role for the LP, which can be activated by MBL, in renal IRI (10-12). To study the contribution of the LP on C-activation by hypoxic human PTEC, these cells were incubated with NHS in the presence or absence of an inhibiting monoclonal antibody to MBL (Fig. 5A). Inhibition of MBL did not affect C3 or C4 deposition, indicating that the LP is not involved. In line with these findings, blockade of the LP using D-Mannose, a ligand for MBL, did not affect C3 or C4 deposition (Fig. 5B). To show that D-Mannose is able to inhibit the LP of complement, we applied an ELISA system with mannan-

coated plates which specifically activate the LP. C-activation in this assay could be completely blocked using D-Mannose, while L-Mannose, which does not bind MBL, had no effect (Fig. 5C). Together these data show that inhibition of the LP had no effect on C-activation by hypoxic human PTEC, indicating that there is no involvement of the LP.

IgM binds to hypoxic human PTEC and contributes to classical pathway activation

The studies above indicate that hypoxic human PTEC exclusively activate the CP in human serum. To obtain more detailed insight in the mechanisms of CP activation by hypoxic human PTEC, we assessed the effect of inhibition of the CP using a monoclonal antibody directed against the C1q-globular head domains. Blockade of the CP using this antibody resulted in a dose-dependent inhibition of both C3 and C4 deposition on hypoxic HK-2 cells (Fig. 6A) and primary PTEC (data not shown). Moreover, incubation of hypoxic HK-2 cells in C1q-deficient serum resulted in an almost complete abrogation of C3 and C4 deposition and reconstitution with purified C1q restored C-activation (Fig. 6B).

The CP can be activated by binding of C1q to different cellular ligands, but also to cell-bound immunoglobulins like IgM. Indeed, we could show a dose-dependent binding of purified IgM to hypoxic human cells (Fig. 7A). Exposure of hypoxic human PTEC to serum depleted of IgM resulted in an almost complete abrogation of C3 deposition, whereas reconstitution of

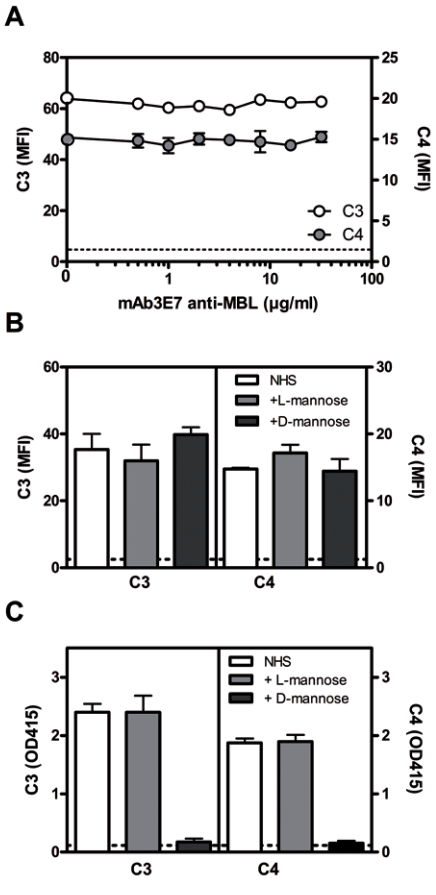

Figure 5. No involvement of lectin pathway of complement. The lectin pathway was studied by (a) incubating hypoxic HK-2 cells with 5% NHS with an increasing dose of blocking antibodies to MBL (mAb 3E7). White dots represent C3 and grey dots C4 deposition. Besides, hypoxic HK-2 cells (b) or mannan coated plates (c) were incubated with NHS in the presence or absence of 200 mM L-mannose (light grey bar) or D-mannose (dark grey bar) after which C3 and C4 deposition was determined. White bars represent the condition with NHS only. Results given are the mean (+ SD) MFI of triplicate cultures and are representative of 3 independent experiments.

this serum with purified IgM fully restored C-activation. (Fig. 7B), indicating a major role for IgM and subsequent C1q on C-activation by hypoxic human PTEC. In contrast to human PTEC, incubation of hypoxic mouse PTEC with serum derived from RAG -/- mice, which lack immunoglobulins, still resulted in extensive C-activation (Fig. 7C) to a similar level as normal mouse serum.

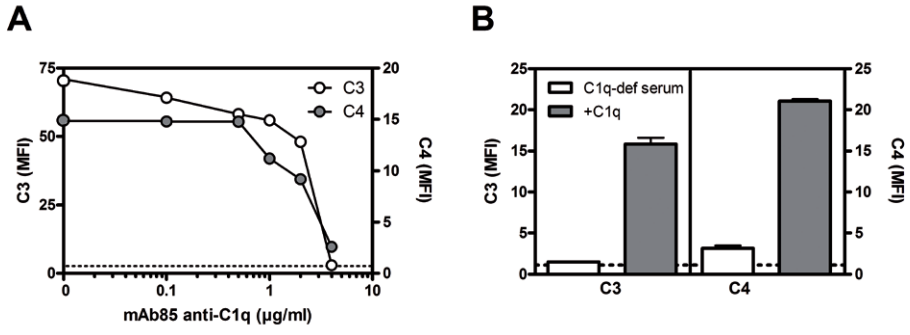

Figure 6. Prominent role for the classical pathway of complement. To study the contribution of the classical pathway, hypoxic HK-2 cells were incubated with 5% NHS with an increasing dose of blocking antibodies to C1q (mAb 85), after which C3 (white dots) and C4 (grey dots) deposition was determined. (a) Hypoxic HK-2 cells were incubated with 5% C1q-deficient serum (white bars), or this serum was reconstituted with purified C1q (100 µg/ml)(grey bar) (b). Results given are the mean (+ SD) MFI of triplicate cultures and are representative of 3 independent experiments.

Recent studies have shown that the binding of IgM to apoptotic cells is mediated for a major part by lyso-phosphatidyl derivatives and that this binding can be blocked using phosphorylcholine (27;28). Indeed, C-activation by hypoxic human PTEC was blocked significantly by 20 mM phosphorylcholine (Fig. 7D), suggesting that natural IgM antibodies can bind to phospholipid neo-epitopes exposed on hypoxic cells and subsequently function as a focus of CP activation by hypoxic human PTEC.

Correlation between IgM levels and complement activation by hypoxic human PTEC

Eleven sera derived from healthy individuals all induced significant C3, C4 and C5b-9 deposition on hypoxic HK-2 cells, compared to normoxic cells (Fig. 8A-C). To determine whether there was an association between IgM binding and classical C-activation we assessed in parallel the binding of IgM and the deposition of C3, C4 and C5b-9 on hypoxic PTEC (Fig. 8D-F). We found a significant correlation between IgM binding and C4 (r^2=0.643), C3 (r^2=0.572) and C5b-9 deposition (r^2=0.570), indicating that IgM binding to hypoxic human PTEC plays an important role in activation of the CP of complement after hypoxic stress.

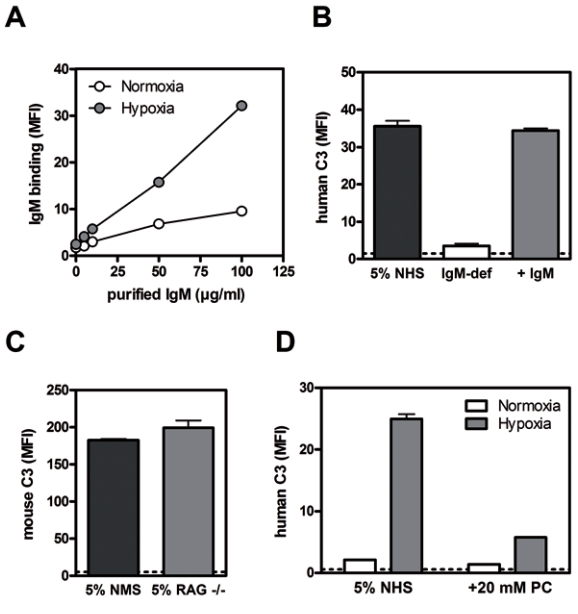

Figure 7. In contrast to mouse PTEC, complement activation by hypoxic human PTEC is IgM-dependent. To establish if complement activation was dependent on bound immunoglobulins, the binding of purified IgM (a) to normoxic and hypoxic HK-2 cells was assessed. To study functional consequences of IgM binding, hypoxic HK-2 cells were incubated with 5% IgM-deficient serum. As a control serum was reconstituted with purified IgM (100 µg/ml) (b). IgM-dependent complement activation by mouse PTEC was studied by incubating hypoxic IMPTEC with 5% NMS or RAG -/- serum after which C3 deposition was determined (c). Results given are the mean (+ SD) MFI of triplicate cultures and are representative of 3 independent experiments. Dotted lines represent background staining. Hypoxic HK-2 cells were incubated with 5% NHS in the presence of 20 mM phosphorylcholine (PC) and C3 deposition was determined (d). Results given are the mean (+ SD) MFI of triplicate cultures and are representative of 2 independent experiments.

DISCUSSION

Several lines of evidence support an important role for the AP of C-activation by proximal tubular cells in *in vivo* mouse models of renal IRI. However, it is still unclear whether similar pathways of C-activation are involved in the human setting. Therefore we studied hypoxia-induced C-activation by human and mouse PTEC. Complement activation by hypoxic mouse PTEC was mainly dependent on the AP, compatible with the *in vivo* observations. In contrast, hypoxic human PTEC primarily activated complement through the CP, with a major involvement of natural IgM antibodies. These results reveal important species-specific differences which might have important implications for complement directed therapies in renal IRI.

As an *in vitro* model of renal IRI, both primary human PTEC and HK-2 cells were cultured under hypoxic conditions and reoxygenated in human serum. This resulted in extensive C-activation by the tubular cells and deposition of C4 and C3

up to the level of C5b-9. Complement activation could be completely abrogated using EDTA, showing that the deposition of complement factors on the hypoxic cells was the result of activation of the complement system.
In our model, normoxic human PTEC showed some deposition of complement, as described before (29), which is partially mediated through binding of properdin (19). Nevertheless, in our model C-activation was markedly increased

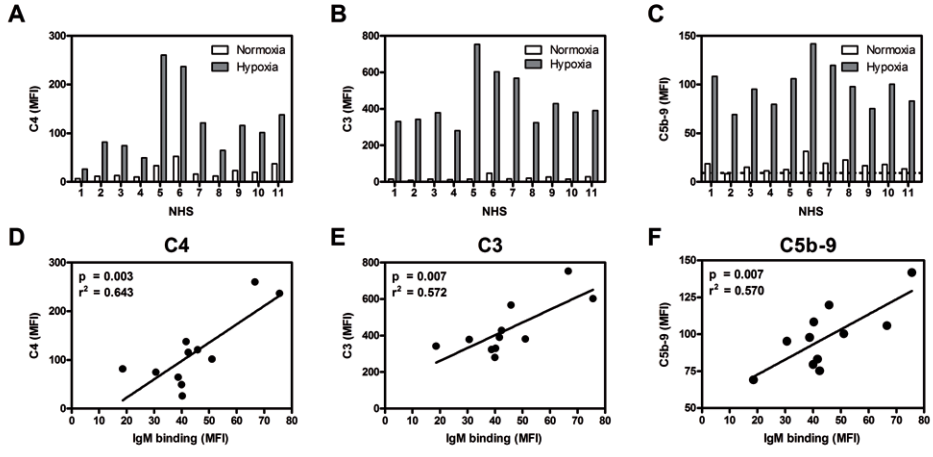

Figure 8. Hypoxia induces IgM-mediated complement activation. Normoxic (white bar) or hypoxic (grey bar) HK-2 cells were incubated with 5% serum derived from eleven different healthy volunteers, and C4 (a), C3 (b) and C5b-9 (c) deposition was determined. Simultaneously, binding of IgM was measured by FACS analysis and correlated to the C4 (d), C3 (e) and C5b-9 (f) deposition. P values and correlation coefficients are given in the graphs.

as a result of hypoxia/reoxygenation compared to the basal level of C-activation. Furthermore, using MgEGTA which blocks the CP and LP, C-activation could be completely abrogated to levels of complement deposited on normoxic PTEC, indicating that the increased deposition of complement on hypoxic cells does not depend on the AP.
Studies with MBL knockout mice have shown a protective effect of MBL deficiency in the setting of renal IRI (11), and also renal deposition of MBL has been demonstrated (10). Moreover, we have previously demonstrated that renal allograft recipients with low MBL levels show a better graft survival (30). However, we could not show a role for MBL in our *in vitro* model using hypoxic human PTEC. Blocking antibodies to MBL or inhibition with D-mannose did not have any effect on C-activation. Although C-activation in the ischemic kidney is largely localized to the tubular epithelium, low grade C-activation on the endothelium could result in activation of the endothelium and extravasation of serum constituents in the interstitium which could lead to C-activation on

tubular epithelial cells. Therefore we hypothesize that MBL is mainly involved in LP activation on endothelial cells as shown before (31;32), or has effector functions that are still unknown.

Using C1q-depleted serum or blocking antibodies to C1q we could reduce C-activation by hypoxic human PTEC to basal levels indicating that the complement system is mainly activated via the CP. Interestingly, C-activation by hypoxic cells that were annexin-V and PI positive, and thus were excluded from analysis, also occurred via the CP, suggesting that hypoxia-induced apoptotic PTEC expose similar ligands as the hypoxic, but still viable cells.

It is known from several studies that late apoptotic and necrotic cells bind natural IgM antibodies which will lead to activation of the CP (27;33). We could also demonstrate such a binding of IgM to hypoxic PTEC. Using IgM-deficient serum we showed that C-activation by hypoxic cells also occurs via binding of IgM, probably via binding of IgM to phosphorylcholine residues exposed on hypoxic cells as shown by inhibition of C-activation using phosphorylcholine. Furthermore, we could show a high correlation between IgM binding and C4, C3 and C5b-9 deposition using sera from different donors, suggesting a prominent role for IgM in activation of the CP. It has been proposed that I-PLA2 activation during apoptosis promotes the exposure of membrane lysophosphatidylcholine leading to binding of natural IgM antibodies and subsequently C-activation(34). It is tempting to speculate that a similar process may be involved on hypoxic human PTEC.

Data from studies in mice indicate that C-activation due to IRI in skeletal muscle (13), heart (14), intestine (35) and limb (28) occurs through binding of natural IgM. However, in renal IRI in mice it has been shown that immunoglobulins do not play a role (17). These findings are in line with reports showing that C4-deficient mice were not protected against renal IRI (9), so it appears that in mice IRI can proceed independently of C4 and immunoglobulins. To confirm this in our model we used mouse PTEC which were rendered hypoxic and were incubated with normal mouse serum supplemented with MgEGTA to block CP and LP activation. Additionally we also used serum from RAG -/- mice which lack immunoglobulins. Indeed, C-activation still proceeded in the absence of immunoglobulins or an active CP and LP, indicating that the AP plays an important role in mice. These data are in contrast to hypoxic human PTEC which primarily activate the CP via IgM but in agreement with *in vivo* studies in mice (7;8). Also in a pig model, it was recently shown that the CP and LP of complement were involved in renal IRI (12). Importantly, these authors showed reduced ischemic injury when pigs were treated with C1INH, a specific inhibitor of both the CP and LP (36;37). Interestingly, this study (12) also demonstrated that these two pathways were activated in renal transplant recipients suffering from delayed

graft function (DGF). The co-localization of C4d with both C1q and MBL in graft biopsies obtained from these patients indicated that both these pathways were activated on peritubular capillaries, within the interstitium, and on the glomerular endothelium.

The potential difference in the mechanisms of C-activation by mouse and human cells, as presented in the current manuscript, has important implications for the interpretation of experimental data obtained in mice. To successfully develop therapeutic interventions targeted towards C-activation, it is essential to establish the validity of murine data relative to what takes place in the human situation. Because this study is limited to an *in vitro* model, further studies are needed to delineate the role of natural IgM and complement in the human situation following renal I/R.

From the results above, we conclude that hypoxia-induced C-activation by human PTEC primarily occurs via the CP of complement, which is dependent on the binding of IgM. This is in contrast to hypoxic mouse PTEC which primarily activate the AP of complement. Together these data provide new clues about the pathways of complement that should be targeted after renal IRI in humans, however further studies in humans are needed.

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CHAPTER 3

TRANSPLANTATION 2013; 95:816-20

Acute but transient release of terminal complement complex after reperfusion in clinical kidney transplantation

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ABSTRACT

Background: Ischemia/reperfusion (I/R) injury has a major impact on kidney graft function and survival. Animal studies have suggested a role for complement activation in mediating I/R injury, however results are not unambiguous. Whether complement activation is involved in clinical I/R injury in humans is still unclear.
Methods: In the present study, we assessed the formation and release of C5b-9 during early reperfusion in clinical kidney transplantation in both living, brain dead and cardiac dead donor kidney transplantation. By arteriovenous measurements and histological studies, local terminal complement activation in the reperfused kidney was assessed.
Results: There was no release of sC5b-9 from living donor kidneys, nor was there a release of C5a. In contrast, instantly after reperfusion, there was a significant but transient venous release of soluble C5b-9 from the reperfused kidney graft in brain dead and cardiac dead donor kidney transplantation. This short-term activation of the terminal complement cascade in deceased donor kidney transplantation was not reflected by renal tissue deposition of C5b-9 in biopsies taken 45 minutes after reperfusion.
Conclusions: This systematic study in human kidney transplantation shows an acute but non-sustained sC5b-9 release upon reperfusion in deceased donor kidney transplantation. This instantaneous, intravascular terminal complement activation may be induced by intravascular cellular debris and hypoxic or injured endothelium.

INTRODUCTION

Ischemia/reperfusion (I/R) injury is an inevitable consequence of organ transplantation and a major determinant of patient and graft survival (1-3). Current therapy is supportive and there are no specific therapeutical options yet. The pathophysiology of I/R injury is complex and incompletely understood. The innate immune system has been suggested to play an important role in potentiating an injurious reaction upon reperfusion since it is prone to recognize not only pathogens but also 'damaged self' (4).
The complement system is one of the fastest responding basal defense mechanisms of the innate immune system. Activation of either the classical, alternative, or Mannan-binding lectin pathway ultimately leads to the formation of C5b-9, otherwise known as the terminal complement complex or membrane attack complex (MAC). Release of soluble (s)C5-9 has been described in a variety of renal disorders, such as lupus nephritis, Henöch-Schonlein Pupura and aHUS,

and has been shown to be a sensitive marker in assessing disease activity (5-8). Renal I/R affects the endothelial as well as the epithelial compartment and might activate the complement cascade leading to deposition of C5b-9 or release of non-lytic sC5b-9.

Animal studies of renal I/R injury generally show that complement inhibition reduces post-reperfusion damage (9-11). Zhou et al more specifically demonstrated the involvement of terminal complement complex C5b-9 (12). However, in recent rat experiments by our group, inhibition of complement activation did not reduce kidney damage and only 24 hours after reperfusion the first signs of complement activation were observed. Moreover, Mannan-binding lectin itself appears to exert cytotoxic effects on the tubular epithelium early after reperfusion, far before first complement deposition was observed (13). These recent findings raise questions about the contribution of complement activation as initiator of I/R injury.

Despite the extensive number of animal experiments, studies on the involvement of complement in human I/R injury are scarce. Studies in the human heart have suggested a role for complement activation in I/R-induced tissue damage (14,15). However, the diverse studies on experimental anti-complement therapy in human myocardial I/R injury did not lead to major improvements yet (16-21).

To address the recent contradictory findings in animals and the lack of evidence for involvement of complement in human I/R injury, we investigated the role of complement activation in the initiation of clinical renal I/R injury. I/R-induced complement activation may take place in both the tubular and vascular compartment (10,22,23). 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.

METHODS

Patient population

Twenty-four patients undergoing renal allograft transplantation were included for arteriovenous sampling; 8 patients receiving a kidney from a living donor, 9 patients receiving a kidney from a brain dead donor and 7 patients receiving a kidney from a cardiac dead donor (patient and transplantation characteristics are in table 1), as previously described (29). Brain dead and cardiac dead donors together were referred to as deceased donors. For technical reasons (renal vein sampling) only patients receiving a left kidney were included. In another 33

patients (13 living donor, 10 brain dead donor, and 10 cardiac dead donor kidney recipients) renal biopsies were collected. The study protocol was approved by the local ethics committee, and informed consent was obtained from each patient.

Operation and materials

Kidney transplantations were performed according to local standardized protocol. In living donors minimally invasive nephrectomy was performed and Custodiol® Histidine–tryptophan–ketoglutarate solution (HTK) solution (Tramedico, Weesp, The Netherlands) was used for cold storage of the kidney. Brain dead and cardiac dead donor kidneys were perfused and stored with either University of Wisconsin solution (UW) or HTK. The immunosuppressive regimen was based on tacrolimus or cyclosporine A in addition with mycophenolate mofetil and steroids in all groups.

Arterial and renal venous blood samples were obtained as described before in detail (25). A schematic drawing of the arteriovenous sampling method is shown in figure 1. In short, via a small catheter placed in the renal vein blood aliquots were sampled at 0, 3, 10 and 30 min. after reperfusion. Paired arterial blood samples were obtained at 0, 3, 10 and 30 min. after reperfusion. All samples were collected in tubes containing EDTA and immediately placed on ice. Blood samples were centrifuged (1,550 g, 20 min, 4°C) and the derived plasma was re-centrifuged (1,550 g, 20 min, 4°C) to deplete it from leukocytes and thrombocytes. Material was aliquotted and stored at -70ºC until assayed.

In another 33 patients (13 living donor, 10 brain dead donor, and 10 cardiac dead donor kidney recipients) renal biopsies were collected. A renal cortical biopsy

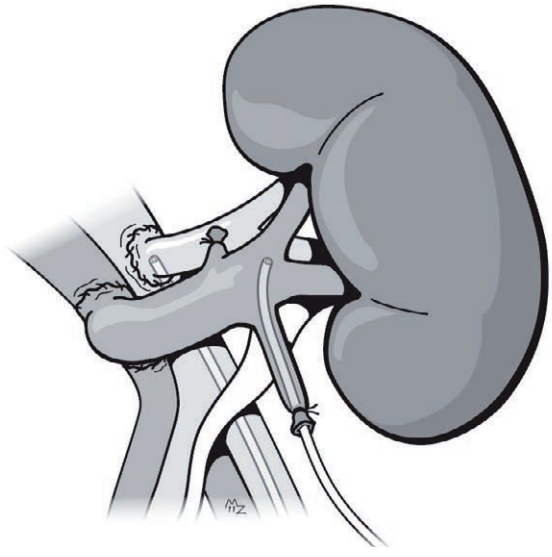

Figure 1. Schematic representation of the arteriovenous sampling method over the reperfused kidney by simultaneous blood collection from the renal artery and vein. *Illustration by Manon Zuurmond© (www.manonproject.com)*

was obtained after cold storage, and a second biopsy of the same kidney was collected 45 minutes after reperfusion.

sC5b-9 and C5a plasma measurements

sC5b-9 and C5a levels were assessed by sandwich ELISA. In short, 96-well ELISA plates (Nunc Bioscience, Belgium) were coated with a monoclonal antibody (mAb) to a neo-epitope on C5b-9 (aE11; Hycult Biotechnology, Uden, Netherlands) or C5a (mAb 2952; Hycult Biotechnology). Plasma was incubated in the coated wells and bound sC5b-9 or C5a was detected with a biotin-labeled mAb to C6 (9C4; in-house made) or C5a (mAb 561; Hycult Biotechnology) respectively, followed by detection with streptavidin–poly-horseradish peroxidase (Sanquin, Amsterdam, The Netherlands). Enzyme activity was detected using 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (Sigma Chemical Co., St. Louis, MO). The optical density was measured at 415 nm using a microplate reader (Model 680; Biorad, Philadelphia, USA). The detection limits for C5a and C5b-9 were 1.95 ng/ml and 0.01 U/ml, respectively.

Immunohistochemistry

Sections (4 µm) of paraffin embedded, formaldehyde fixed biopsies were deparaffinized and treated with 0.1% protease (type XXIV pronase, Sigma) for antigen retrieval. Endogenous peroxidase activity was blocked with 0.1% H_2O_2 and 0.1% NaN_3. Consequently, C5b-9 deposition was assessed using a mAb to a neoepitope on C5b-9 (aE11, Hycult Biotechnology) followed by anti-mouse peroxidase-conjugated EnVisionTM (DAKO, Glostrum, Germany). The staining was visualized using Nova RED (Vector Labs, Peterborough, United Kingdom). Sections were counterstained with hematoxylin (Merck, Darmstadt, Germany). As a positive control, a renal biopsy of a patient with acute graft rejection was used.

Data collection and statistical analysis

Clinical donor data were retrieved from Eurotransplant. Outcome measures were creatinine clearance at 30 days after transplantation, presence and duration of delayed graft function (DGF) and patient and graft survival. DGF was defined as need for dialysis within 7 days post-transplantation. Statistical analysis was performed using SPSS 16.0 statistical analysis software (SPSS Inc, Chicago, Ill). Wilcoxon signed ranks test was used for paired non-parametric data, the Mann-Whitney test for unrelated non-parametric data, i.e. comparison of different donor types. Graph points represent the median and error bars represent the interquartile range. A p-value of less than 0.05 was considered significant.

	LD	BDD	CDD
N	8	9	7
Donor age: mean (SD)	43.9(10.6)	54.1(17.1)	52.7(15.3)
Donor gender (M:F %)	75:25%	44:56%	43:57%
Duration ICU stay in h. (SD)	N/A	126 (211)	107 (139)
Duration of BD in h. (SD)	N/A	14.7(9.7)	N/A
Preservation fluid (n)	HTK (8)	UW (9)	UW(2),HTK (5)
WIT1 in min. (SD)	N/A	N/A	23.1(7.7)
CIT in h. (SD)	3.0(0.3) * #	19.7(6.2)	17.3(2.6)
WIT2 in min. (SD)	34.0(6.3)	33.0(6.1)	34.1(6.4)
Recipient age: mean (SD)	41.1(10.5)	55.1(13.5)	54.0(11.2)
Recipient gender (M:F %)	38:62%	44:56%	71:29%
Preemptive transplantation (n)	1	0	0
Creatinine clearance day 30 in ml/min (SD)	73.3 (20.5) * #	49.3(15.3) #	27.1(10.3)
DGF (%)	0%	56%	86%
DGF: dialysis after transplantation in days (SD)	0 (0) * #	7.0 (5.3) #	17.2 (7.2)

Table 1: Transplantation and outcome characteristics of patients undergoing arteriovenous measurements. Groups undergoing living donor (LD), brain dead donor (BDD) and cardiac dead donor (CDD) kidney transplantation were compared. Intensive care unit (ICU) stay: the period the donor was admitted to intensive care unit, BD: the total duration of brain dead of the donor, WIT1: first warm ischemia time, CIT: cold ischemia time, WIT2: second warm ischemia time, DGF: delayed graft function. *p<0.05 compared to BDD, # p<0.05 compared to CDD.

RESULTS

Donor and transplant characteristics

Recipient and donor age and gender were similar in living donor, brain dead donor and cardiac dead donor groups (Table 1). As expected, warm and cold ischemia times differed between the groups, with shorter cold ischemia times in living donor kidney transplantation. The immunosuppressive regimen did not differ between groups. A significantly higher rate of delayed graft function (DGF) was observed in brain dead donor and cardiac dead donor as compared to living donor kidney transplantation. Brain dead donor and cardiac dead donor

transplantation were equal in occurrence, but not in duration of DGF. All kidneys were still functioning at 1 year post-transplantation, except for one kidney (the recipient was not compliant with immunosuppressive medication).

Early release of soluble complement complex C5b-9 from the kidney into the circulation

Activation of the terminal complement cascade during reperfusion was assessed by measuring the release of soluble (s)C5b-9 complex from the kidney by arteriovenous measurements (Figure 1). Immediately at reperfusion there was an acute but transient release of sC5b-9 from deceased donor kidneys, which was not observed from living donor grafts (LD p=0.46, BDD p=0.011, CDD p=0.028; Figure 2). There was no release of sC5b-9 at later timepoints, at 3 (LD p=0.31, BDD p=0.77, CDD p=0.06), 10 (LD p=0.48, BDD p=0.68, CDD p=0.08) or 30 minutes (LD p=0.12, BDD p=0.78, CDD p=0.74) after reperfusion. Soluble C5a, as an alternative sign of complement activation, was measured in arteriovenous samples in living donor kidneys. In accordance with sC5b-9 measurements, there was no difference in arterial and renal venous C5a levels (p=1.00 at 5 minutes, p=0.29 at 30 minutes after reperfusion, data not shown). Differences in the net

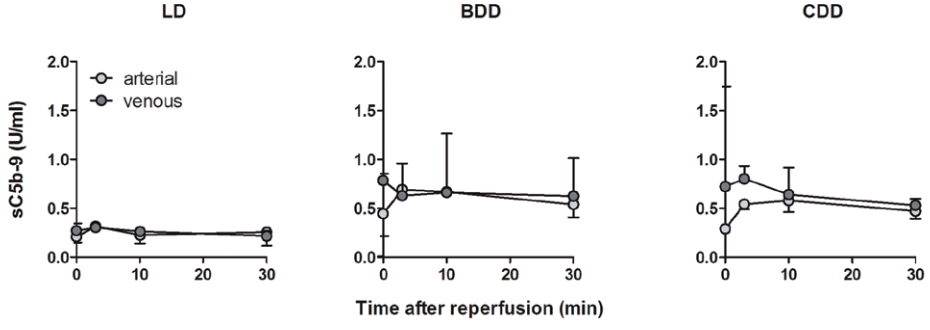

Figure 2. sC5b-9 concentration in arterial and venous blood samples in living donor (LD), brain dead donor (BDD) and cardiac dead donor (CDD) kidney transplantations in the first 30 minutes of reperfusion. There was a significant release of sC5b-9 within sec after reperfusion (first time point) from BDD kidney grafts (n=9, p=0.011) and from CDD grafts (n=7, p=0.028), but not from LD grafts (n=8, p=0.46). At later time points there was no significant difference anymore. Graphs show median and interquartile range.

release of sC5b-9 from the kidney for the total of 30 minutes were assessed by comparing arterial and venous area under the curve. For living (p=0.87) and brain dead donor grafts (p=0.26) no net release was observed from the kidney. Cardiac dead donor kidneys, however, showed a significant release of sC5b-9 from the kidney for the total first half hour after reperfusion (p=0.018). Baseline (t=0) arterial values of sC5b-9 were not different between LD, BDD and CDD recipients (p=0.08).

No increase in local, tissue-bound complement complex C5b-9 after reperfusion

The acute but transient release of soluble C5b-9 into the circulation might be accompanied by local C5b-9 deposition in the kidney, and local deposition could contribute to the absence of circulating C5b-9. Therefore, presence and localization of C5b-9 in pre- and post-reperfusion kidney biopsies was assessed. Whereas the renal biopsy of acute rejection tissue showed extensive C5b-9 positivity, staining for C5b-9 revealed no vascular or tubular depositions of C5b-9 before or after reperfusion in any of the three donor types (Figure 3).

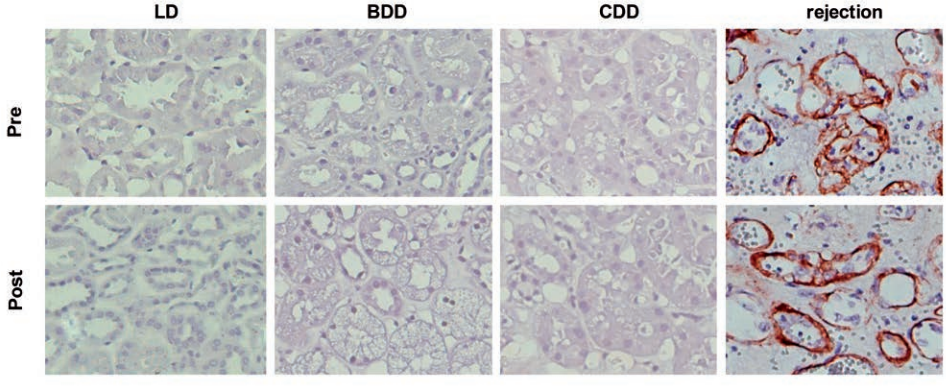

Figure 3. Representative sections showing distribution of C5b-9 staining in a pre- and post-transplantation biopsies of living donor (LD), brain dead donor (BDD) and cardiac dead donor (CDD) kidneys. There were no C5b-9 depositions in any of the biopsies (LD n=13, BDD n=10, CDD n=10). In contrast, the positive control biopsy of a kidney graft with acute rejection showed massive peritubular and tubular C5b-9 depositions. Original magnification, x200.

DISCUSSION

I/R injury is one of the main causes of delayed graft function in transplantation. Studies in mice have suggested a predominant role for complement activation in renal I/R injury. However, in our recent study we show that not complement activation, but rather direct cytotoxic effects of circulation derived Mannan-binding lectin initiate tissue injury in rat renal I/R experiments (13). Studies on timing and role of complement activation in human renal I/R injury are scarce and inconclusive. Therefore, we set out to assess the role of terminal complement activation in the initiation of renal I/R injury in humans. Our data show that there is acute, non-sustained terminal complement activation upon reperfusion in deceased donor kidney transplantation.

We concentrated on measurement of sC5b-9 as it is the common end-point of both the classical, alternative and Mannan-binding lectin pathway of the complement cascade. Moreover, in mice it is suggested that specifically C5b-9 is essential in the induction of tubular damage in renal I/R injury (12). By measuring arteriovenous differences over the reperfused organ, we were able to obtain very specific data on local venous release of sC5b-9 from the human kidney. In a previous study involving living donor kidney transplantations only, we found no release of sC5b-9, but rather an early and vast release of interleukin-6 from the kidney (24). In the current study the group is expanded with kidneys from brain dead and cardiac dead donors which are more severely affected by I/R. We show that from these deceased donor kidney grafts sC5b-9 is indeed released directly after reperfusion, indicative of intravascular terminal complement activation. Since sC5b-9 is released transiently, directly after reperfusion, this may result from a wash out effect. The complement system may be triggered upon encounter with intravascular cellular debris accumulated during the cold ischemic period or by encounter with hypoxic or injured endothelium (23,25). Studies in other human organs, such as the heart confirm complement activation in I/R injury (14,15), although these observations may be influenced by complement activating effects of the cardiopulmonary bypass machine (26).
Besides the intravascular sC5b-9 formation, C5b-9 could be formed locally in the tissue without any release into the circulation. To assess this tubular activation, tissue content and distribution of C5b-9 was assessed in kidney biopsies collected before and after reperfusion. There was no deposition of C5b-9 in the kidney after reperfusion in both living and deceased donor kidney transplantation. This is confirmed by a study of Haas et al. where in post transplantation biopsies no complement depositions as consequence of reperfusion were detected either (27). In contrast, renal tissue of a patient with acute graft rejection showed extensive C5b-9 deposition in the tubular compartment.
Finally, the possibility remained that the complement cascade is activated in living donor kidneys as well, without leading to terminal complement activation. Therefore, release of C5a from the reperfused kidney was assessed because C5a is more upstream in the complement cascade than the terminal complex C5b-9 is. In agreement with sC5b-9 measurements, there was no C5a release from living donor kidneys. This excludes complement activation after reperfusion in living donor kidneys and also excludes early involvement of C5a, which has also been ascribed a harmful role in I/R injury (9).
A limitation of our study was the fact that the sampling time was restricted to maximally 30 minutes following reperfusion. Although complement activation in this study was only observed instantly after reperfusion, mouse experiments show membrane attack complex elements C6 and C9 later on, at 12 and 24

hours after reperfusion, respectively (28). Furthermore, one may consider the sample size as a limitation. However, as the goal of this study was to assess the basic pathophysiological role of complement activation in I/R injury, instead of correlating findings to clinical outcomes, small patient numbers were sufficient. Finally, only cortical biopsies could be collected, as deeper puncture holds a high risk of bleeding and calycal injury and was considered unsafe.

In summary, this systematic study in human kidney transplantation shows acute, non-sustained intravascular terminal complement activation during early reperfusion of deceased donor kidney grafts, likely to be initiated by contact with intravascular cellular debris and injured or hypoxic endothelium.

ACKNOWLEDGEMENTS

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CHAPTER 4

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Pitfalls in urinary complement measurements

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ABSTRACT

Local activation of the complement system has been associated with ischemia/reperfusion injury following kidney transplantation and tubular injury under proteinuric conditions. The soluble terminal complement complex sC5b-9 is a stable end-product of the complement cascade, and as such a promising urinary biomarker. In the early post-transplant period we found high urinary levels of sC5b-9, significantly correlating with the degree of proteinuria, suggesting activation of filtered complement components at the tubular epithelial surface of the kidney. However, when mimicking proteinuria *in vitro* by exposing serum (or blood) to urine (both negative for sC5b-9), we found extensive generation of sC5b-9 in urine. This process was inhibited by EDTA, confirming activation of the complement system. In conclusion, although sC5b-9 is an attractive urinary biomarker, one should be aware of the risk of extra-renal complement activation independent of a renal contribution. This may be of special interest when measuring urinary sC5b-9 following kidney transplantation in which procedure-related (microscopic) hematuria and proteinuria are common.

BRIEF COMMUNICATION

Complement activation at the tubular epithelial surface of the kidney, which lacks several important complement regulators (CD46, CD55) (1), is considered to be a mediator of tubular injury in the proteinuric condition. In proteinuria, complement proteins, which normally are retained in circulation, are able to pass the glomerular filter barrier, end up in the tubular lumen and are activated by the unprotected epithelial surface of the tubuli (2-4). To this end, the detection of soluble (s)C5b-9 in urine is widely considered as a clinical indicator of tubular complement activation (5-7).

The complement system, a set of circulating proteins of the innate immune system that forms a biochemical cascade, is activated by the binding of complement recognition molecules (e.g. C1q, MBL or properdin) to their respective target (e.g. pathogens or apoptotic cells). Three activation pathways have been recognized, namely the classical, lectin and alternative pathway, which all converge at the level of complement component C3. Subsequent downstream activation of the complement cascade leads to formation of the lytic terminal complement complex C5b-9, which is able to damage and lyse target cells. Recently, it was shown that complement activation and deposition of C5b-9 on tubular epithelial cells is mediated by binding of properdin, the initiator of the alternative pathway of complement (8).

Increased glomerular permeability to large plasma proteins (proteinuria) is common in the early period following renal transplantation, with a prevalence of 15% to 30% at 1 year post-transplantation (9). Activation of filtered or locally produced complement components is likely to be involved in tubulotoxicity of proteinuria (2;3). Complement activation products indeed are detectable in the urine of patients with different proteinuric renal diseases (10;11) and are believed to be one of the possible candidates mediating tubular injury in the proteinuric condition (2;3). However, in the days after transplantation, not only glomerular damage may be responsible for proteinuria, but also a procedure-related (microscopic) hematuria.

Table 1. Donor, recipient and graft characteriscrtics.

	All (n = 24)
Donor age (years)	47 ± 16
Donor gender (% female)	58
Recipient age (years)	52 ± 14
Diuresis preTx (patient numbers)	17
Cold ischemia time (hours)	17,5 ± 5,0
Warm ischemia time 1 (min)	20,3 ± 6,6
Warm ischemia time 2 (min)	29,0 ± 7,2
Delayed graft function (patient numbers)*	17
One-year patient survival (%)	91,7%
One-year graft survival (%)	88.5%

*Cases are categorized as DGF when the serum creatinine level increases, remains unchanged, or decreases by less than 10 % per day immediately after surgery during 3 consecutive days within the first week

In the present study we confirm the relation of urinary sC5b-9 and proteinuria in a renal transplantation cohort and we investigated the possibility whether in proteinuric urine sC5b-9 can be generated independent of a renal contribution. In a cohort of deceased cardiac dead donors (Table 1), high levels of urinary sC5b-9 were detected (Fig 1A) at day 2 after transplantation, which decreased

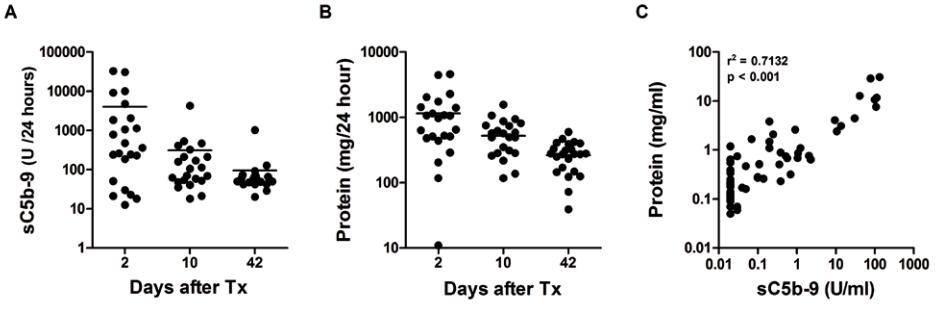

Figure 1. Urine from recipients of a renal allograft were collected at day 2, 10 and 42 after transplantation and assessed for sC5b-9 (A) and proteinuria (B). Levels of sC5b-9 at day 2, 10 and 42 (C) were correlated to the measured proteinuria.

slowly after 10 and 42 days. Although urinary output was still variable at day 2, almost all patients showed normal diuresis at day 10. Most patients suffered from proteinuria (Fig 1B), and the degree of proteinuria strongly correlated with the urinary sC5b-9 levels (Fig 1C).

To assess whether this observed complement activation may be an extra-renal phenomenon, the proteinuric condition was mimicked *in vitro*. Seven urine samples from healthy volunteers, all free of sC5b-9, were incubated with a

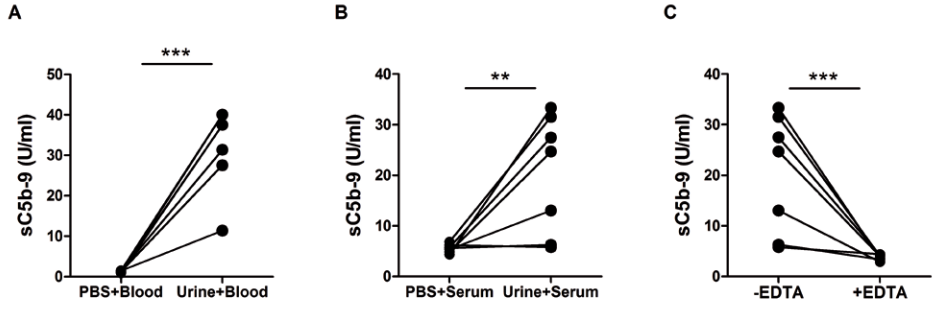

Figure 2. Blood (A) or serum (B, C) from seven healthy volunteers was 1:4 diluted in the corresponding urines or PBS and incubated for 60 minutes at 37°C followed by assessment of sC5b-9. Additionally, 10mM EDTA was added to the serum/urine to block complement activation (C). ** P < 0.01; *** P<0.001.

small amount of freshly drawn blood from the corresponding individuals, which strikingly resulted in extensive generation of sC5b-9 in urine (Fig 2A). In contrast, levels of sC5b-9 remained low when the same amount of blood was added to PBS instead of urine, indicating that complement can be activated in urine *ex vivo*, independent of any renal contribution. To assess whether this effect was due to the presence of blood cells (12) (e.g. in hematuric conditions), similar dilutions of human serum in urine were tested. This also resulted in an extensive generation of sC5b-9 (Fig 2B), ruling out any effect of blood cells on the observed complement activation.

The active process of complement activation in the urine samples *in vitro* was confirmed by the complete abolishment of complement activation when EDTA was added before incubation (Fig 2C). Since EDTA chelates calcium and magnesium needed for complement activation, measured urinary sC5b-9 must be formed by active complement activation *in vitro* in urine.

The urinary protein content in the transplantation patients varied from 0 to 10 mg/ml (Fig 1C). In an additional experiment, this range was approached by serially diluting serum (normal serum protein content is 60-80 mg/ml) 4 to 32 times (Fig 3A). Even when serum was 32 times diluted in urine (reflecting a urinary protein content of 1.8-2.5 mg/ml), significant levels of sC5b-9 could be

detected. This indicates that also in less severe proteinuric conditions, sC5b-9 can be generated without any renal contribution.
Urine, even from healthy subjects, often contains viable or apoptotic renal cells and cellular debris (13;14), as turnover or injury to epithelial cells lining the urinary tract results in shedding of these cells into urine which potentially could lead to activation of complement proteins present in proteinuric urines (15;16). To investigate such an involvement, the urine was filtered with a 0.2 µm filter to remove cells and cellular particles which possibly remained after centrifugation. Next, the corresponding serum was added to the filtered or unfiltered urine to allow sC5b-9 generation (Fig 3B). Removal of remaining cells and cellular debris reduced the sC5b-9 generation in the urine by half, suggesting that sC5b-9 generation in proteinuric urine is partially caused by cellular debris and apoptotic epithelial cells. The remaining complement activation observed after filtration,

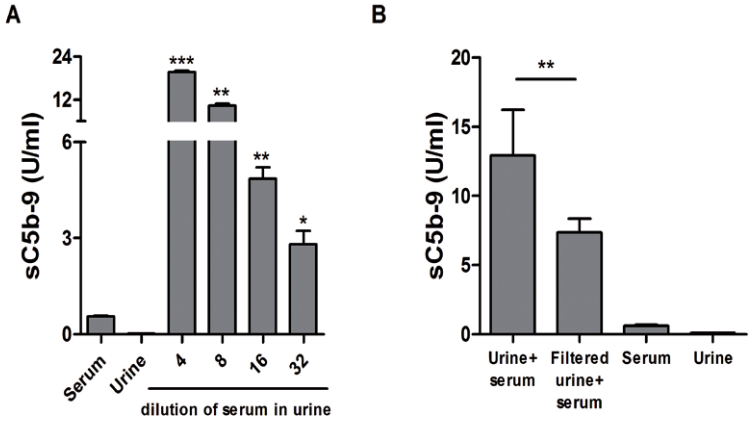

Figure 3. Serum from a healthy volunteer was serially diluted in the corresponding urine and incubated for 60 minutes at 37°C followed by assessment of sC5b-9. Represented data are true sC5b-9 concentrations, uncorrected for dilution. sC5b-9 levels in the diluted serum were compared to undiluted serum. * P < 0.05; ** P < 0.01; *** P<0.001 (A).
Urine from 4 healthy volunteers was filtered with a 0.2 µm filter to remove any remaining cells or cellular debris. Subsequently, corresponding sera were 1:4 diluted in the filtered and unfiltered urine for 60 minutes after which sC5b-9 generation was assessed. ** P < 0.01 (B).

may be explained by high urinary levels of ammonia (17;18) or low urinary pH (12), which have been shown to favor urinary complement activation.
After renal transplantation, proteinuria is a common event, with a prevalence of 15% to 30% at 1 year after transplantation (9). Here, we show that after renal transplantation, urinary sC5b-9 can be detected in the majority of renal allograft recipients and significantly correlates with the degree of proteinuria. This would classically be interpreted as activation of complement by the tubular epithelial

surface (8;19). However, in this study we show that, independent of a renal contribution, presence of blood or serum constituents in urine form healthy subject could lead to complement activation and generation of sC5b-9 *in vitro*. This implies that following transplantation, proteinuria and procedure-related (microscopic) hematuria may cause urinary complement activation resulting in high urinary sC5b-9 levels. Centrifugation or filtration following collection of patient urines would not prevent this, since urinary complement activation most probably occurs in the urinary tract.
We would like to conclude that urinary sC5b-9 measurement is troubled by extra-renal or even *ex vivo* complement activation in case of hematuric or proteinuric conditions, rendering the implications and clinical relevance of measured urinary sC5b-9 rather unpredictable.

METHODS

Patient population

Twenty-four patients undergoing a renal allograft transplantation receiving a kidney from a deceased cardiac death donors in the period between August 2005 and September 2006 were recruited (Table 1). The study protocol was approved by the local ethics committee, and informed consent was obtained from each patient.

Operation and materials

Donor kidney transplantations were performed according to the local protocol. From allograft recipients, urine was collected at consecutive days after transplantation. Urine samples were centrifuged at 2500g at 4° Celsius for 10 minutes, aliquotted and stored at −80° C for later complement measurements.

Urine measurements

Soluble C5b-9 was measured by ELISA using an antibody to a neoepitope on C5b-9 (AE11) (20). Total protein was measured by a colorimetric method.

Urinary complement activation

Whole blood or serum from 7 healthy volunteers was 1:4 or serially diluted in their corresponding urine or PBS, and incubated for 60 minutes at 37°C (mimicking the hematuric or proteinuric condition, respectively). Additionally, the process of complement activation was prevented by adding 10 mM EDTA. To investigate any involvement of remaining renal cells or cellular debris, urine from 4 healthy volunteers was filtered with a 0.2 µm filter (GE Healthcare, Little

Chalfont, UK). Subsequently, corresponding sera were 1:4 diluted in the filtered and unfiltered urines and incubated for 60 minutes at 37°C. After incubation, in all samples further complement activation was blocked by adding 10mM EDTA. Samples were then immediately processed for sC5b-9 measurement as described.

Statistics

Correlation analysis between variables was performed by linear regression and the significance of differences was calculated by a Mann-Whitney test using GraphPad Prism software. Differences with $P<0.05$ were considered significant.

ACKNOWLEDGEMENTS

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CHAPTER 5

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Mannan-binding lectin mediates renal ischemia/reperfusion injury independent of complement activation

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ABSTRACT

Ischemia/reperfusion injury (IRI) remains a major problem in renal transplantation. Clinical studies have identified that high serum levels of Mannan-binding lectin (MBL), the initiator of the lectin pathway of complement activation, are associated with inferior renal allograft survival. Using a rat model, we identified an entirely novel role for MBL in mediating renal IRI. Therapeutic inhibition of MBL was protective against kidney dysfunction, tubular damage, neutrophil and macrophage accumulation, and expression of proinflammatory cytokines and chemokines. Following reperfusion, exposure of tubular epithelial cells to circulation-derived MBL resulted in internalization of MBL followed by the rapid induction of tubular epithelial cell death. Interestingly, this MBL-mediated tubular injury was completely independent of complement activation since attenuation of complement activation was not protective against renal IRI. Our identification that MBL-mediated cell death precedes complement activation strongly suggests that exposure of epithelial cells to MBL immediately following reperfusion is the primary culprit of tubular injury. In addition, also human tubular epithelial cells *in vitro* were shown to be susceptible to the cytotoxic effect of human MBL. Taken together, these data reveal a crucial role for MBL in the early pathophysiology of renal IRI and identify MBL as a novel therapeutic target in kidney transplantation.

INTRODUCTION

Ischemia/reperfusion injury (IRI) is a key event in clinical conditions such as infarction, sepsis and organ transplantation. Restoration of blood flow to ischemic tissue paradoxically exacerbates tissue damage by initiating a cascade of inflammatory events including release of pro-inflammatory cytokines and chemokines, recruitment of leukocytes and activation of the complement system (1-3). Numerous clinical and experimental studies have shown that renal IRI profoundly impacts short-and long-term graft survival following kidney transplantation, and is strongly associated with delayed graft function (DGF) and clinical morbidity and mortality (3-5). DGF results in oliguria, increased allograft immunogenicity and risk of acute rejection episodes (5) and is associated with extensive loss of the cortico-medullary proximal tubular epithelial cells (PTEC), often referred to as acute tubular necrosis (ATN). To date, no effective therapy or treatment for renal IRI in the clinic exists.

Renal ischemia is accompanied by a reduction of membrane-bound complement regulators on PTEC, rendering these cells susceptible to complement activation

following reperfusion (6). Activation of the complement system has been documented as one of the hallmarks of renal IRI (6-8). The complement system is a central component of innate immunity and consists of three pathways of activation, namely the classical, alternative and lectin pathway. The complement cascade is activated by the binding of recognition molecules, such as C1q and MBL, to their respective target, e.g. pathogens, apoptotic or necrotic cells (9). All three pathways converge at the level of complement factor C3, which is cleaved and subsequently deposits as C3b on target cells resulting in opsonization and clearance by phagocytic cells. In absence of effective clearance, further activation of the complement cascade leads to generation of the membrane attack complex (MAC) C5b-9 and lysis of target cells.

MBL is the major recognition molecule of the lectin pathway of complement activation. Single-nucleotide polymorphisms in both structural and regulatory parts of the MBL gene have been found to lead to large interindividual variations in the concentration of functional MBL in serum (0-4 μg/ml) (10). Previously, we have shown that low pre-transplantation levels of MBL are associated with better graft survival after deceased-donor kidney transplantation. These findings identify MBL as a potential risk factor for graft and patient survival in renal transplantation (10;11). In rodents, MBL exists in two distinct isoforms, namely MBL-A and –C (12). Nevertheless, specific inhibition of MBL-A in myocardial infarction in rats has been shown to be effective in reducing post ischemic damage (13). Modulation of the complement system has been recognized as a promising strategy in drug discovery, and a large number of therapeutic modalities have been developed (14;15), suggesting that therapeutic strategies could be employed to improve clinical outcomes following renal IRI. In the present study we examined 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.

METHODS

Animals

The Animal Care and Use Committee of the Leiden University Medical Center approved all experiments. Eight-week-old male Lewis rats (200–250 gram) purchased from Harlan (Horst, The Netherlands), were housed in standard laboratory cages and were allowed free access to food and water throughout the experiments. Unilateral ischemia was induced by clamping of the left renal pedicle for 45 minutes using a bulldog clamp (Fine Science Tools, Heidelberg,

Germany). During clamping the contralateral kidney was removed. Sham-treated rats had identical surgical procedures except for clamping of the left kidney but including removal of the contra-lateral kidney. Before induction of ischemia, rats were treated with anti-rat MBL-A mAb (IgG1; P7E4; 1 mg/kg), anti-rat C5 mAb (IgG2b; 18A; 20 mg/kg) or as a control anti-human fibronectin mAb (IgG1; 1 mg/kg or 20 mg/kg respectively) (13;16). To deplete rats (n=5 per group) of C3, cobra venom factor (CVF, 2 mg/kg; Sigma-Aldrich, Zwijndrecht, The Netherlands) or PBS as a control, was administered intraperitoneally 19 hours before induction of ischemia. To prevent CVF-induced C5 activation, rats were pretreated with anti-rat C5 mAb (18A; 20 mg/kg).

Assessment of kidney function

Renal function was determined by measuring creatinine and urea in serum samples using standard autoanalyzer methods by our hospital research services.

Renal histology

Histological evaluations were performed in a blinded manner by a single pathologist. Formalin-fixed kidneys embedded in paraffin were sectioned and stained with silver by standard methods.

Renal immunostaining

Rat kidney sections (4 μm) of snap-frozen kidneys were air dried and acetone-fixed. Neutrophil (PMN) and macrophage (Mph) accumulation was assessed using digitonin (DIG)-conjugated mAbs against CD43 (W3/13) and CD68 (ED-1) respectively, followed by horseradish peroxidase (HRP)-conjugated sheep anti-DIG (Roche Diagnostics, Mannheim, Germany). The staining was visualized using Nova RED (Vector Labs, Peterborough, United Kingdom). Rat C3 deposition was assessed by direct staining using a fluorescein isothiocyanate (FITC)-conjugated rabbit polyclonal to rat C3 (made in-house). Quantification of immunohistochemistry was performed in a blinded manner by assessing 10 consecutive high power fields (HPFs; magnification ×200) of the outer medulla and cortico-medullary junction on each section. Using image J software, the positive area in each image (expressed in pixels) was quantified. Deposition of rat or human MBL was assessed using anti-rat MBL (mAb 14C3) or anti-human MBL (mAb 3E7; Hycult Biotech, Uden, The Netherlands) respectively, followed by HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc). The staining was visualized using tyramide labeled with tetramethylrhodamine-5-(and 6)-isothiocyanate (Sigma-Aldrich). Nuclei were stained using Hoechst (Molecular Probes, Leiden, The Netherlands). Fluorescent micrographs were made using confocal laser scanning microscopy (LSM 510, Zeiss, Germany).

RNA isolation and Real-Time PCR

Rat kidney RNA was extracted from snap frozen renal tissue and purified using an RNeasy Mini isolation Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Specific rat primers for Kidney injury molecule (KIM)-1, Neutrophil gelatinase-associated lipocali (NGAL), Macrophage chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-2 and ribosomal protein S-15 (RPS-15, housekeeping gene), were designed using Oligo Explorer and obtained from Biolegio (Nijmegen, The Netherlands). The primer sequences were as follows: KIM-1 (forward, 5'- TACCAACATCAACCAGAGTCTC -3'; reverse, 5'-ACATAGAAGCCCTTAGTCGG-3'), NGAL (forward, 5'-CGATGAACTGAAGGAGCGATTC -3'; reverse, 5'- TGGCAACAGGAAAGATGGAG - 3'), MCP-1 (forward, 5'-GCCCAGAAACCAGCCAAC -3'; reverse, 5'- GCCAGTGAATGAGTAGCAGC - 3'), MIP-2 (forward, 5'-TCAATGCCTGACGACCCTAC-3'; reverse, 5'-GAGCCCATGTTCTTCCTTCC - 3') and RPS-15 (housekeeping gene): forward 5' CGTCACCCGTAATCCACC-3' and reverse 5'-CAGCTTCGCGTATGCCAC -3'). PCR was performed using SYBR Green PCR mastermix (Biorad). Data were analyzed using Gene Expression Analysis for iCycler Real-Time PCR Detection System (Biorad). Expression of each gene was normalized against mRNA expression of the housekeeping gene Rsp-15. RT PCRs were performed in duplicate.

Immunohistochemistry

Functional activity of the lectin pathway in rat serum was assessed by detection of C3 deposition on mannan-coated plates as previously described (17). Hemolytic activity of rat serum was assessed in an AP50 hemolytic assay as previously described (18).

Cell culture

Immortalized human PTEC (HK-2) (18) were grown as previously described (19). Primary PTEC from pre-transplant biopsies (20) and human umbilical vein endothelial cell (HUVEC) (21) were isolated and cultured as described. Primary cells were used at passage 3 or less.

Flow cytometry

Cells were fixed using 1% paraformaldehyde (Pharmacy) and permeabilized using 0.1% saponin (Sigma-Aldrich). Internalized MBL was detected using anti-human MBL (mAb 3E7; Hycult Biotech) followed by RPE-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, USA). MBL staining was assessed using a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA). MBL binding and subsequent internalization was inhibited using 10 mM EDTA (Sigma Aldrich).

Fluorescence microscopy

Cells were fixed using cold methanol (Pharmacy) and stained with anti-human MBL (mAb 3E7; Hycult Biotech) and rabbit polyclonal anti-human protein disulfide isomerase (PDI, Cell Signaling Technology, Danvers, MA, USA) followed by alexa-488 conjugated goat anti-mouse IgG (Molecular Probes) and alexa-568 conjugated goat anti-rabbit IgG (Molecular Probes), respectively.

Viability assays

Cell viability was assessed by FACS analysis using propidium iodide (PI; Molecular Probes, Leiden, The Netherlands) and Annexin V-FITC (VPS Diagnostics, Hoeven, The Netherlands). Cell viability was also assessed by counting Hoechst-positive, but PI-negative cells using fluorescence microscopy. Inhibition studies were done using D- or L-mannose (Sigma-Aldrich) or a blocking antibody to MBL (3F8).

Preparation of human MBL

MBL was purified from human serum as previously described (22).

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM) and subjected to nonparametric statistical analysis using one- and two-factor ANOVA or Mann-Whitney test using GraphPad Prism software. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Inhibition of MBL preserves renal function after ischemia/reperfusion

To therapeutically target MBL in renal IRI we used a mAb to MBL-A (P7E4) which previously has been shown to effectively reduce post-ischemic damage following myocardial infarction in rats (13). Five minutes before induction of ischemia rats were treated with anti-MBL-A resulting in a transient inhibition of lectin pathway activity that normalized 24 hours afterwards (Fig 1A), while classical pathway activity was unaffected (data not shown). The lectin pathway activity in rats receiving the isotype-matched control antibody was unaffected (Fig 1A). Induction of ischemia for 45 minutes followed by reperfusion resulted in a marked impairment of renal function in control treated rats as measured by serum levels of creatinine (Fig 1B) and urea (Fig 1C) with levels rising within 24 hours and remaining high until 72 hours after reperfusion. In contrast, rats receiving the blocking antibody to MBL-A were protected against renal dysfunction, with serum levels of creatinine and urea close to the normal range (Fig 1B and C).

Furthermore, renal dysfunction in control-treated rats was accompanied by a significant decrease in weight at 72 hours after reperfusion, compared to sham or anti-MBL treated rats (Fig 1D). Despite the transient inhibition of MBL-A, the protection against renal IRI was almost complete, indicating that MBL-A exerts its harmful effects relatively short after reperfusion.

Anti-MBL treatment reduces tubular injury and leukocyte infiltration

Ischemia/reperfusion resulted in extensive ATN (Fig 2A) leading to tubular cast formation and obstruction of tubules (Fig 2E). These events were significantly reduced by inhibition of MBL-A (Fig 2B,F), with a reduction of 70% in ATN (Fig

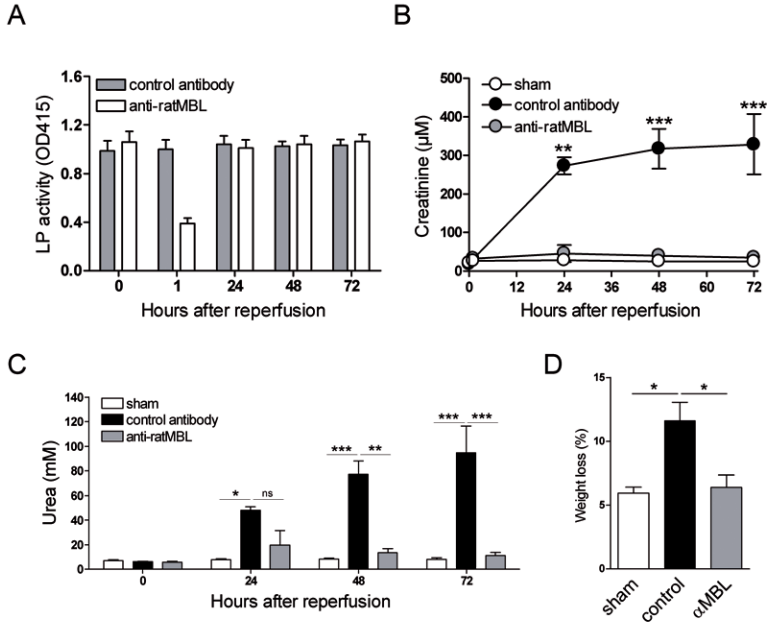

Figure 1. Inhibition of MBL-A protects against renal ischemia/reperfusion injury. Before induction of unilateral ischemia and removal of the contralateral kidney, rats received anti-MBL-A or an isotype-matched control mAb (1 mg/kg). Before and following reperfusion blood samples were drawn at consecutive time points to determine lectin pathway activity (A). Renal function was assessed by measuring serum creatinine (B) and urea (C) levels. After 72 hours of reperfusion rats were weighted and compared to pre-IRI weight to calculate weight loss (D). Data are shown as mean ± SEM, n= 6 per group. The comparison between groups is indicated by asterisks. * P < 0.05; ** P<0.01;***P<0.001.

2C) and 66% in tubular cast formation (Fig 2G). Assessment of renal mRNA levels of KIM-1 and NGAL, both markers of tubular injury, revealed significantly lower KIM-1 expression (Fig 2D) in the anti-MBL treated group. NGAL expression had a tendency to decrease (Fig 2H).

Tubular injury after reperfusion was accompanied by extensive infiltration of PMN (Fig 3A) and Mph (Fig 3E) in the outer medullary zone. Inhibition of MBL-A significantly reduced accumulation of PMN (Fig 3B, C) and Mph (Fig 3F, G), and chemokine expression of MIP-2 (Fig 3D) and MCP-1 (Fig 3H).

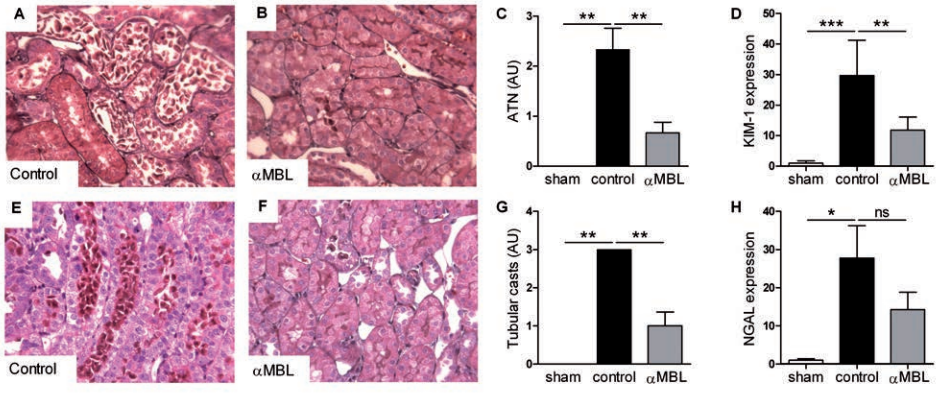

Figure 2. Tubular injury is significantly reduced by inhibition of MBL-A. Silver staining of renal sections from control (A, E) and anti-MBL (B, F) treated rats 72 hours following reperfusion showing ATN (A) and tubular cast formation (E) (original magnification x200). ATN (C) and tubular cast formation (G) were analyzed semi-quantitatively. Renal mRNA expression of injury markers KIM-1 (D) and NGAL (H) in renal tissue at 72 hours following reperfusion was assessed. Data are shown as mean ± SEM (n= 6 per group). The comparison between groups is indicated by asterisks. * P < 0.05; ** P<0.01;***P<0.001.

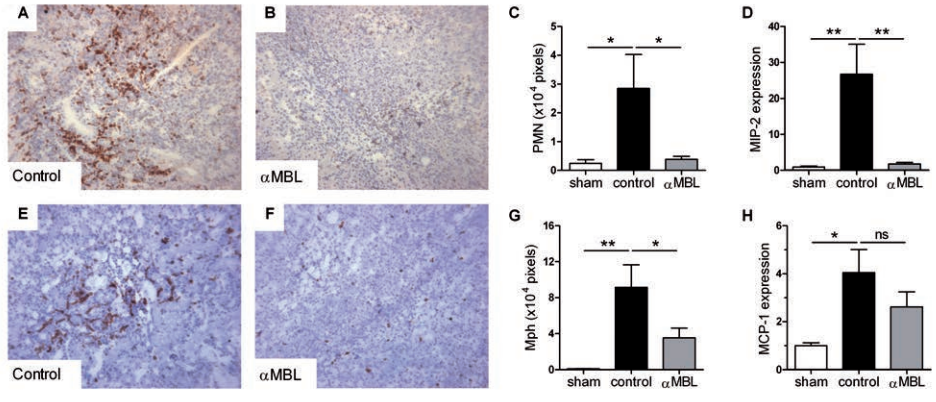

Figure 3. Macrophage and neutrophil accumulation was significantly reduced by inhibition of MBL-A. Renal sections from control (A, E) and anti-MBL (B, F) treated rats 72 hours following reperfusion were stained for PMN (A, B) and Mph (E, F) (original magnification x200). Infiltrates of PMN (C) and Mph (G) were quantified using digital image analysis. Renal mRNA expression of the chemokines MIP-2 (D) and MCP-1 (H) in renal tissue at 72 hours following reperfusion was assessed. Data are shown as mean ± SEM (n= 6 per group). The comparison between groups is indicated by asterisks. * P < 0.05; ** P<0.01.

Induction of reperfusion injury is independent of complement activation

In mice it has been shown that the MAC (C5b-9) plays an important role in the induction of tubular injury following renal IRI (6). To dissect the role of C5 (which is downstream of MBL and C3) in our renal IRI model, we applied an anti-rat C5 antibody (mAb 18A), which was previously shown to significantly reduce injury in a rat myocardial IRI model (16). Injection of anti-rat C5 before induction of ischemia resulted in complete inhibition of the terminal pathway of complement for at least 24-48 hours, whereas the control antibody had no effect (Fig 4A). However, despite this relative long-term inhibition of C5, rats were not protected against renal dysfunction, showing equally high creatinine levels as compared to control-treated rats (Fig 4B). Furthermore, there was no significant difference in tubular injury (Fig 4C) and cast formation (Fig 4D) suggesting that the tubular injury is not induced by C5 activation in this model. Moreover, assessment of Mph and PMN influx revealed an extensive influx of inflammatory cells in both the anti-C5 and control-treated group (Fig 4E, F).

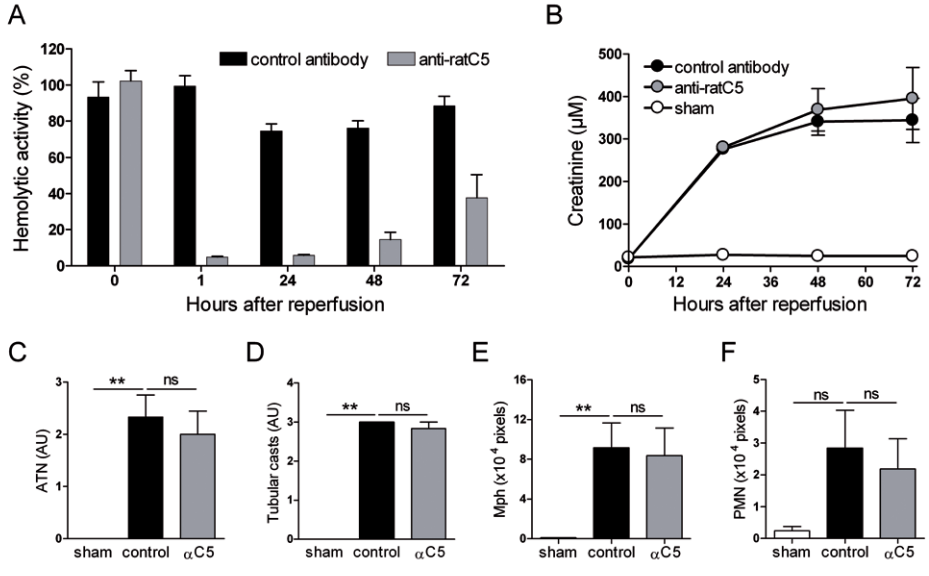

Figure 4. Inhibition of C5 does not ameliorate renal ischemia/reperfusion injury. Before induction of unilateral ischemia and removal of the contralateral kidney rats received anti-rat C5 or a control mAb (20 mg/kg). Before and after reperfusion blood samples were drawn at consecutive time points to determine the inhibitory capacity of anti-rat C5 mAb by measuring serum hemolytic activity in an AP50 hemolytic assay (A). Renal function was assessed by measuring serum creatinine levels (B). Using silver staining, ATN (C) and tubular cast formation (D) was assessed semi-quantitatively at 72 hours after reperfusion. Renal sections were stained for Mph (E) and PMN (F) infiltrate and analyzed quantitatively. Data are shown as mean ± SEM (n= 6 per group). The comparison between groups is indicated by asterisks. ** P<0.01.

Since inhibition of MBL-A was shown to be very effective in reducing renal IRI, whereas inhibition of C5 was not, we set out to dissect the role of C3. Depletion of C3 was achieved using cobra venom factor (CVF) and was preceded by administration of anti-C5 to prevent excessive C5 cleavage and generation of C5a. Injection of anti-C5 abrogated terminal complement activity (Fig 5A) and subsequent administration of CVF completely depleted the pool of C3 for at least 48 hours after reperfusion (Fig 5B). Despite depletion of C3 and inhibition of C5, this did not result in protection against renal IRI but even tended to an increased

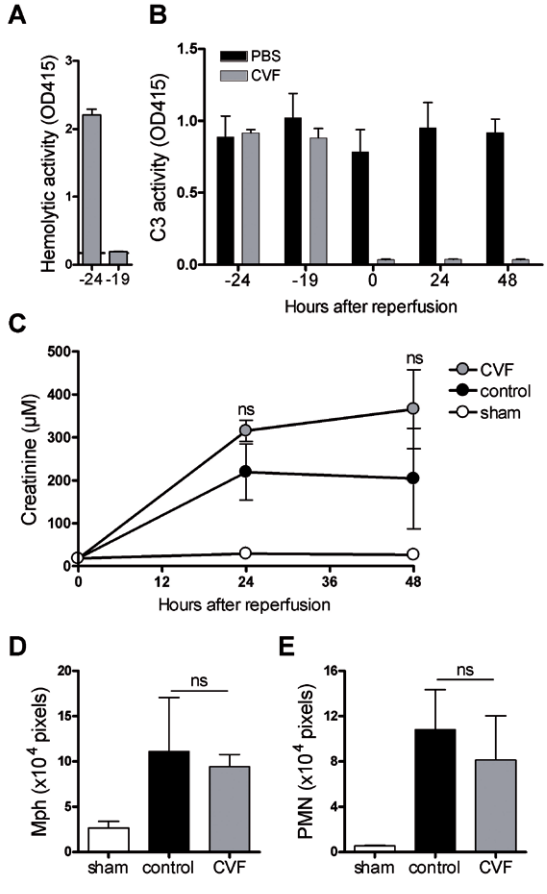

Figure 5. Depletion of C3 does not protect against renal ischemia/reperfusion injury. Twenty-four hours before induction of ischemia, all groups received anti-rat C5 (20 mg/kg). Five hours afterwards and 19 hours before induction of ischemia, rats received CVF to deplete C3. Sham-operated rats also received CVF, while the control group received PBS. Blood samples were drawn at consecutive time points to determine hemolytic activity in an AP50 assay (A) and C3 activation in a lectin pathway activation assay (B). Renal function was assessed by measuring serum creatinine levels (C). Renal sections following 48 hours of reperfusion were stained for Mph (D) and PMN (E) infiltrate and analyzed quantitatively. Data are shown as mean ± SEM (n= 5 per group).

impairment of renal function (Fig 5C). CVF did not affect kidney function, since sham operated rats which also received CVF and were uninephrectomized, showed stable serum creatinine levels (Fig 5C). Also the influx of Mph (Fig 5D) and PMN (Fig 5E) at 48 hours was not reduced, indicating that not solely C3a or C5a were responsible for the influx of inflammatory cells into the renal tissue.

Complement is activated in a late phase following renal ischemia/reperfusion and is preceded by deposition of MBL and tubular injury

Taken together, these findings led us to question the contribution of different complement components in the early induction of renal IRI. Therefore, we further investigated the kinetics of tubular injury and complement activation in our model. Signs of ATN, including loss of tight junctions and epithelial sloughing, were early events (within 2-5 hours after reperfusion; Fig 6A) and were accompanied by deposition of MBL (Fig 6B). At this stage however, deposition of C3 (Fig 6C) and C5b-9 (Fig S1) was completely absent, implying that tubular injury could not be caused by activation of complement and generation of the lytic C5b-9 complex. Only from 24 hours onwards, C3 deposition was observed on injured MBL-positive tubular cells (Fig 6D). This delayed appearance of C3 could not be explained by a change in local expression, as C3 expression even decreased from 5 hours onwards (Fig S2). Together, these findings suggest a differential role for MBL and complement activation in renal IRI.

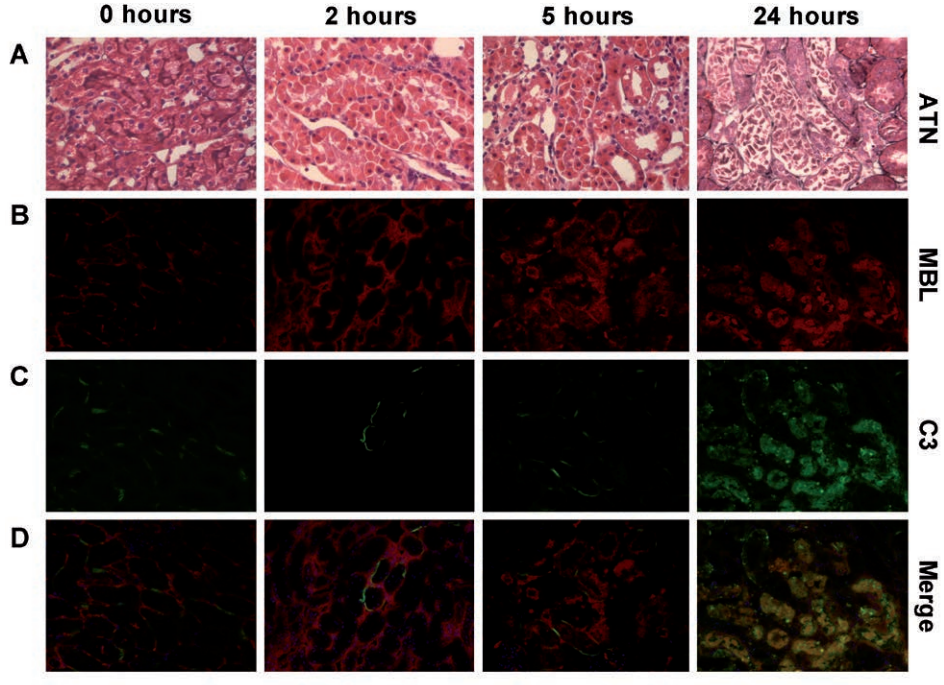

Figure 6. Complement is activated in a late phase following reperfusion and is preceded by deposition of MBL and tubular injury. Silver staining (A) of renal sections from rats (n=6 per group) sacrificed prior to, 2, 5 or 24 hours after reperfusion showing early loss of tight junctions and cell adherence at 2 and 5 hours followed by ATN at 24 hours. Renal sections were triple stained (D) for MBL (B; red), C3 (C; green) and Hoechst (blue) (original magnification x200).

Upon reperfusion MBL leaks from circulation and is internalized by tubular epithelial cells

This prompted us to seek for alternative effector functions than activation of complement by which MBL potentially could injure tubular cells. First we studied the localization and deposition of MBL following reperfusion in more detail. Staining of kidneys for rat MBL-A, showed minor staining in the peritubular capillaries of sham operated rats (Fig 7A), reflecting circulating MBL. In contrast, 2 hours after reperfusion we observed an interstitial staining for MBL, surrounding the basolateral side of the tubuli (Fig 7B). Even more, also an intracellular staining of MBL-A in tubular epithelial cells was observed (Fig 7C), suggesting that MBL

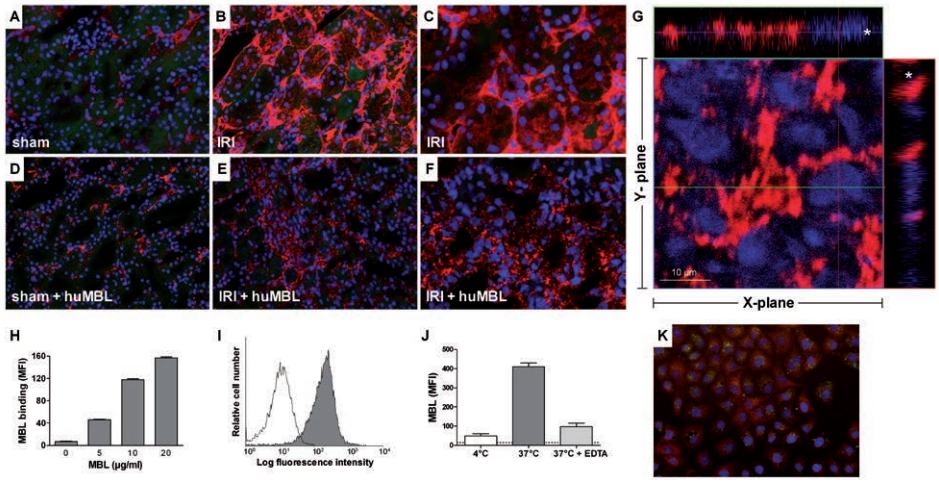

Figure 7. Following reperfusion MBL leaks from circulation and is internalized by tubular epithelial cells. Renal sections from sham operated (A) and ischemic rats (B, C) 2 hours following reperfusion, were stained for rat MBL (red). Nuclei (blue) were stained using Hoechst. To discriminate between locally expressed or circulation-derived MBL, rats were injected with human MBL and sacrificed 3 hours after reperfusion. Sections from the right control kidney (D) or left ischemic kidney (E, F) were stained for human MBL (red). Presence of intracellular human MBL (red) was confirmed using confocal scanning laser microscopy (G), showing MBL staining in the same Z-plane (marked by asterisks) near the nuclei (blue). To study the interaction of human MBL with human PTEC *in vitro*, HK-2 cells were incubated with purified human MBL (0-20 µg/ml) for 30 minutes at 4°C, followed by flow cytometry analysis (H, I). To study internalization of MBL, PTEC were incubated with 10 µg/ml human MBL at 4 or 37°C and intracellular MBL staining was assessed using flow cytometry (J). Values shown are mean ± SEM of triplicate determinations from a single experiment and are representative of 3 similar experiments. Dotted line represents background staining. Furthermore, internalization of MBL by cultured human PTEC was assessed using fluorescence microscopy (K). Human PTEC (HK-2) were incubated with 10 µg/ml human MBL for 60 min at 37°C and stained (n=5) for intracellular MBL (green), ER-marker PDI (red) and nuclei (blue) (original magnification x200 for A, B, D, E; x400 for C, F, K).

from circulation had been internalized. However, assessment of MBL-A mRNA revealed local MBL-A expression in the kidney (Fig S2), leaving the option that intracellular MBL might be the result of local production.
To discriminate between locally expressed or circulation-derived MBL, we intravenously administered human MBL (50 µg) shortly after reperfusion resulting in a serum concentration of 2 µg/ml human MBL (data not shown). Specific staining for human MBL of the reperfused kidney 3 hours following reperfusion revealed an intense staining surrounding (Fig 7E) and inside tubular cells (Fig 7F), which was absent in the contralateral control kidney (Fig 7D). Intracellular MBL was further demonstrated using confocal microscopy (Fig 7G), showing MBL staining (red) in the same plane (Z-plane) in between the nuclei (blue). This confirms that upon reperfusion of the ischemic kidney, MBL from circulation leaks into the interstitium where it is internalized by tubular cells. This staining was most pronounced in the cortico-medullary border, the region mostly damaged.
To further study whether MBL is able to interact with tubular cells, we cultured and exposed human PTEC to human purified MBL *in vitro*, which resulted in dose-dependent binding of MBL as determined by flow cytometry (Fig 7H, I). To study internalization of MBL by tubular cells as observed *in vivo* following reperfusion, human PTEC were incubated with MBL at 4 or 37°C followed by an intracellular FACS staining (Fig 7J). We observed an increased staining at 37°C, indicating that MBL was indeed internalized. Incubation of MBL in presence of EDTA, which chelates calcium needed for binding, largely inhibited binding and subsequent internalization of MBL. These data were confirmed by immunofluorescence using an intracellular staining method, showing a perinuclear staining pattern of human MBL (Fig 7K), indicating that binding of MBL is followed by internalization. Together these data show that MBL specifically binds to PTEC which is followed by internalization.

MBL induces tubular epithelial cell death

To study the functional consequence of MBL binding and internalization, human PTEC were incubated with different concentrations of purified MBL (0-10 µg/ml) for different time points. Strikingly, already within 5 hours of MBL incubation (10 µg/ml) alterations in cell morphology were observed. Tubular epithelial cells start shrinking and rounding, followed by detachment from the plate within 24 hours of incubation with MBL (Fig 8A), resembling the situation observed *in vivo* upon IRI. FACS staining of PTEC for PI and Annexin V after 24 hours of incubation with MBL (10 µg/ml) revealed that most cells were in an early (25%) or late apoptotic (44%) phase (Fig 8C), whereas untreated cells (90%) remained viable (Fig 8B). This cytotoxic effect was time- and dose-dependent and was

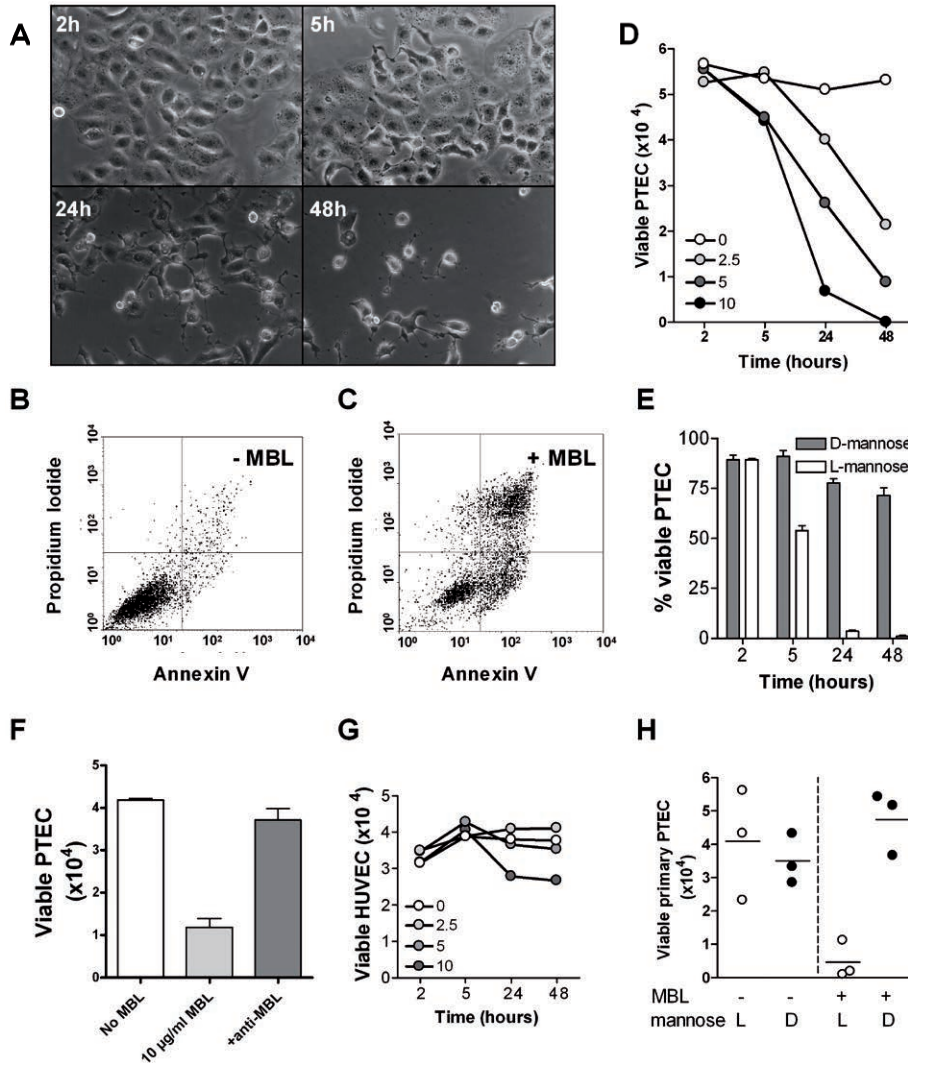

Figure 8. MBL induces tubular epithelial cell death. Human PTEC (HK-2) were incubated with 2.5, 5 or 10 (A) µg/ml human MBL for 2-48 hours. Wells were photographed (A, magnification ×200) and cell viability was assessed by counting Hoechst-positive, but PI-negative cells in the well using fluorescence microscopy (D). Cell viability at 24 hours incubation with 0 (B) or 10 (C) µg/ml MBL was assessed by FACS analysis using propidium iodide (PI) and Annexin V-FITC. HK-2 cells were incubated with 10 µg/ml MBL in the presence of D- or L-mannose (200 mM) for 2-48 hours (E) and cell viability was determined using PI and Annexin V. HK-2 cells were incubated with 10 µg/ml MBL in the absence or presence of an inhibitory mAb to MBL (25 µg/ml; 3F8) for 24 hours (F). Primary HUVEC were incubated with 2.5, 5 or 10 µg/ml human MBL for 2-48 hours (G). Primary PTEC were incubated with 10 µg/ml MBL in the presence of D- or L-mannose (200 mM) for 24 hours (H). Cell viability was assessed by counting Hoechst-positive, but PI-negative cells (F-H).

accelerated at higher doses of MBL (Fig 8D), and even at a physiologic MBL concentration of 2.5 μg/ml this effect was clearly present. To show specificity, MBL was preincubated with D-mannose (a ligand for MBL) which resulted in a very effective inhibition of MBL-induced cell death, as assessed with Annexin V and PI (Fig 8E). In contrast, incubation with L-mannose, which does not bind MBL, showed no inhibition indicating that binding through the lectin domain of MBL is involved in exerting this cytotoxic effect. Prolonged incubation with MBL in the presence of D-mannose still resulted in complete inhibition of cell death, showing that D-mannose not only delayed, but actually prevents MBL-induced cytotoxicity. These data were confirmed using a blocking antibody to MBL (3F8) recognizing an epitope on the carbohydrate recognition domain resulting in an almost complete inhibition of MBL-induced cell death (Fig 8F). The cytotoxic effect was specific for epithelial cells, since HUVEC were not affected by MBL (Fig 8G). All PTEC experiments were performed using a PTEC cell line (HK-2). Therefore, we confirmed these observations by exposing primary human PTEC to MBL, which again resulted in MBL-induced cell death. Also here, this cell death could be prevented by the addition of D-, but not L-mannose (Fig 8H).

DISCUSSION

In the present study we identified a novel role for MBL in the pathogenesis of renal IRI. In a rat model of renal IRI, we demonstrated that therapeutic inhibition of MBL is protective against renal IRI. Following reperfusion, exposure of tubular epithelial cells to circulation-derived MBL resulted in internalization of MBL followed by the rapid induction of tubular epithelial cell death. This MBL-mediated tubular injury was completely independent of complement activation since interference with C3 or C5 was not protective against renal IRI. MBL-mediated cell death preceded complement activation strongly suggesting that exposure of epithelial cells to MBL immediately following reperfusion is the primary culprit of tubular injury, and not the lytic C5b-9 complex.

Several studies, mainly performed in mice, have shown an important role for complement in the induction of renal IRI. A study using C3, C5 and C6 deficient mice showed a predominant role for C5b-9 in renal IRI (6), and also inhibition of C5 with monoclonal antibodies was protective (23). In contrast to these data, others have shown that gene knock-out (24), inhibition (24) or depletion of C3 (25) was not protective against renal IRI. In our model deposition of C3 and C5b-9 following reperfusion was observed, although this was in a relatively late phase (24 hours onwards). A similar late kinetics of C5b-9 deposition has also been shown in a mouse IRI model (23), although this model was characterized

by an early increase in deposition of C3 (2 hours). We did not observe early C3 deposition, indicating possible differences in kinetics and pathogenesis of renal IRI among species. Differences in organ size and metabolism between species could play a role in the observed differences, given that in mice clamping times of usually 20-25 minutes are needed to induce renal injury, while in rats 45-60 minutes of clamping time is needed to induce substantial IRI. Also, difference in relative strength of complement activation pathways could be of influence. In this respect, it has been shown that serum complement activity even among rat strains are varying (26), explaining why in certain rat strains complement activation could be more involved.

Despite the fact that complement activation was not involved in the induction of renal IRI in our model, we did find a pivotal role for MBL in the pathogenesis of renal IRI, since transient inhibition of MBL-A in the early phase following reperfusion was completely protective. Studies in MBL-deficient mice, which are protected against renal IRI, confirm these findings (27). It is tempting to speculate that these mechanisms might contribute to our previous observation that genetically determined high levels of MBL were an important risk factor for renal graft loss following kidney or simultaneous pancreas kidney transplantation (10;11).

Deposition of MBL has been observed in human biopsies early after transplantation (28) and in pigs following renal IRI (14), and is classically linked to activation of the lectin pathway of complement. Interaction of MBL with meprins expressed on tubular epithelial cells was shown to be the initial step for complement activation (29;30). However, for the first time we here show a differential role for MBL in renal IRI, independent of its capacity to activate the complement system. MBL is able to directly induce renal tubular epithelial cell death in the complete absence of complement activation.

Recently, in intestinal IRI in man it was shown that MBL null alleles were associated with preserved epithelial cell integrity (31). Interestingly, also here no signs of complement activation were observed, suggesting that also in intestinal IRI, MBL might have a cytotoxic effect on epithelial cells.

In oncology, MBL has been shown to bear anti-tumor activity which has been termed MBL-dependent cell-mediated cytotoxicity (32). However, to the best of our knowledge, our studies are the first to identify a direct cytotoxic effect of MBL on non-malignant cells. Recently, an intracellular form of MBL has been described which possibly functions as a cargo transport lectin facilitating ER-to-Golgi traffic in glycoprotein quality control (33). Exogenous MBL internalized by tubular epithelial cells potentially could overload and dysregulate this system leading to induction of epithelial cell death, however more research is required to unravel the exact mechanism involved in MBL-induced tubular cell death.

In summary, our results document the important role of MBL in the early pathogenesis of renal IRI and suggest that therapeutic intervention at the level of MBL could significantly alter the extent of kidney damage following ischemia/reperfusion.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

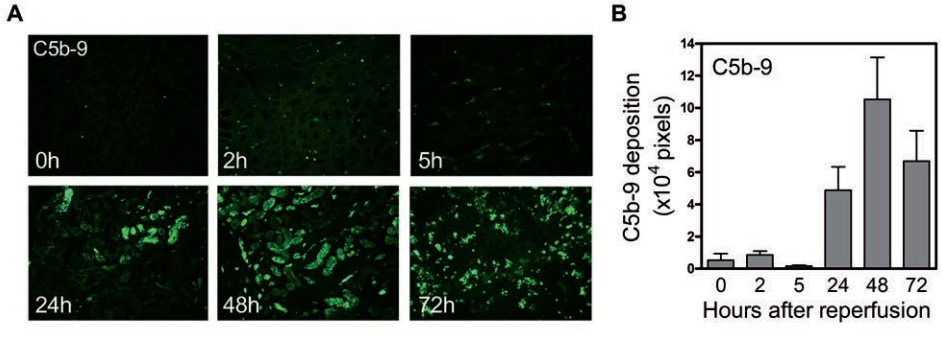

Figure S1. C5b-9 deposits in a late phase following renal ischemia/reperfusion. Deposition of C5b-9 in the cortico-medullary region at consecutive time points after reperfusion (A). C5b-9 deposition was assessed using a mAb to a neoepitope on rat C5b-9 (2A1, Hycult Biotechnology, Uden, The Netherlands) followed by HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). The staining was visualized using tyramide labeled with fluorescein (tyramide-FITC, Sigma-Aldrich-Aldrich). C5b-9 deposition was quantified using digital image analysis (B).

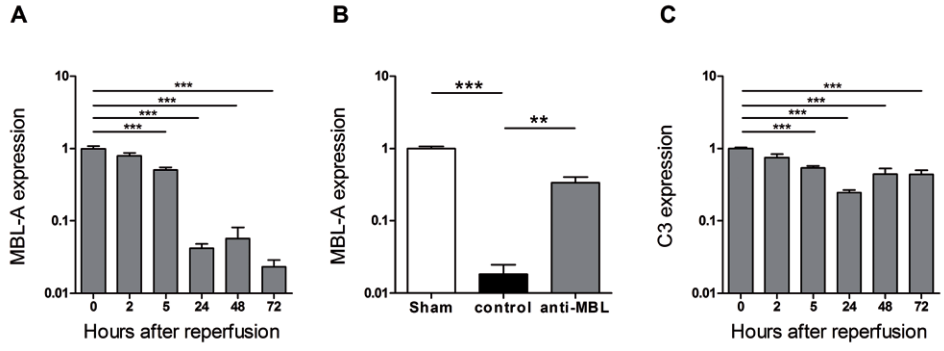

Figure S2. Renal ischemia/reperfusion affects local MBL-A and C3 expression. Renal mRNA expression of MBL-A (A, B) and C3 (C) at consecutive time points after reperfusion in untreated rats (A, C) and rats treated with anti-MBL or control antibody at 72 hours after reperfusion (B) was assessed. Data are shown as mean ± SEM (n= 6 per group). The comparison between groups is indicated by asterisks. * $P < 0.05$; ** $P<0.01$. The primer sequences were as follows: MBL-A (forward, 5'- GGAAACCCTGAAGACTTGC -3'; reverse, 5'- CTGCCTCCATATTTGCCAG - 3') and C3 (forward, 5'- GCCAGCAGCTCTACAATGTG -3'; reverse, 5'- GACTGCCACTTTCCCATAGC- 3').

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CHAPTER 6

IN PREPARATION

Mannan-binding lectin mediates endoplasmic reticulum stress and affects mitochondrial homeostasis in tubular cells following renal ischemia/ reperfusion through GRP78/BIP

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ABSTRACT

Ischemia/reperfusion injury (IRI) is a key event in kidney transplantation and is characterized by tubular epithelial cell injury. Recently, we demonstrated a crucial role for Mannan-binding lectin (MBL) in the pathogenesis of renal IRI. MBL, activator of the lectin pathway of complement, was shown to be directly cytotoxic to tubular cells independent of complement activation.

Here, we demonstrate that exposure of tubular cells to MBL *in vitro* mediates endoplasmic reticulum (ER)-stress. Assessment of ER-stress markers sXBP-1 and CHOP revealed a potent ER-stress response with a twenty- and tenfold induction respectively, accompanied by a strong IL-6 expression. Strikingly, ER-stress was followed by an excessive ATP production, loss of the mitochondrial membrane potential and production of mitochondrial superoxide. Confocal microscopy revealed a strong colocalization of internalized MBL with ER-stress sensor GRP78 and mitochondria, suggesting that interaction of MBL with GRP78 and mitochondria is detrimental to cellular homeostasis.

Assessment of sXBP-1 following renal ischemia/reperfusion in rats revealed an extensive induction of ER-stress within 2 hours, accompanied by an elevated IL-6 expression. Interestingly, assessment of GRP78 protein expression revealed a complete loss of GRP78 in the cortico-medullary region already 2 hours after reperfusion followed by tubular injury and abundant KIM-1 expression within 24 hours. Importantly, therapeutic inhibition of MBL completely prevented loss of GRP78, diminished IL-6 expression and protected against tubular injury.

In conclusion, we demonstrated that MBL mediates ER-stress and affects mitochondrial homeostasis in tubular cells. Interaction with GRP78 might be the mechanism by which MBL mediates tubular injury following renal ischemia/reperfusion.

INTRODUCTION

In kidney transplantation, ischemia/reperfusion (I/R) is an inevitable event and associated with extensive injury to the renal proximal tubular epithelial cells (PTEC). Numerous clinical and experimental studies have shown that renal IRI profoundly impacts short- and long-term graft survival following kidney transplantation, and is strongly associated with delayed graft function and clinical morbidity and mortality (1-3). To date, no effective therapy or treatment for renal IRI in the clinic exists.

Hypoxia/reoxygenation during renal ischemia/reperfusion (I/R) is a complex stress, characterized by interconnected cellular processes such as ATP depletion,

protein misfolding, generation of reactive oxygen species (ROS), and elevation of intracellular calcium. PTEC are highly metabolic active and equipped with a vast amount of mitochondria, and are therefore particularly sensitive to these processes.

The ER is the major intracellular calcium storage site and high lumenal calcium is essential for normal ER function. ATP shortage during ischemia results in a calcium leak from the ER, which impairs the activity of molecular chaperones and maturation of native proteins. These events result in accumulation of mis- and unfolded proteins in the lumen of the ER leading to ER-stress (4-6).

Calcium flux from the ER into the cytosol is buffered to some degree by mitochondrial calcium uptake stimulating mitochondrial ATP production. However, once a continuous increase in cytosolic calcium exceeds the buffering capacity, mitochondria become dysfunctional and mitochondria-mediated apoptotic pathways are activated leading to cell death (7;8). Various adaptive responses to maintain homeostasis and survival are therefore employed, including the activation of the ER-stress response.

The glucose-regulated proteins (GRPs), a family of molecular chaperones and calcium-binding proteins located in ER are induced by ER-stress. Induction of GRPs by ER-stress protects cells against oxidative stress and ischemia-related processes. The concept of ischemic preconditioning is based on this concept where a mild insult is sufficient to induce GRP expression and renders cells tolerant to a subsequent lethal insult (9;10). GRP78, a molecular chaperone also known as BiP or immunoglobulin heavy chain binding protein, is the master modulator of the ER-stress response and under physiologic conditions is associated with three resident ER transmembrane proteins, Upon dissociation and binding of GRP78 to misfolded proteins, these transmembane proteins are activated which leads to a variety of cellular responses to restore normal ER function (4-6).

Recently, we demonstrated a crucial role for Mannan-binding lectin (MBL) in the pathogenesis of renal IRI (11). MBL, an innate immune protein and activator of the lectin pathway of complement, was shown to be directly cytotoxic to tubular epithelial cells independent of complement activation. Upon reperfusion of the ischemic kidney, vascular leakage exposes tubular epithelial cells to circulation-derived MBL, which contributed to tubular injury. Considering the important protective role of GRPs in renal IRI, we hypothesize that MBL might interfere with GRPs leading to tubular cell death.

Here, we demonstrate that renal I/R is accompanied by ER-stress and loss of GRP78 in the most affected cortico-medullary region of kidney. Therapeutic targeting of MBL *in vivo* protects tubular cells located in this region from loss of vital GRP78 and consequent tubular injury. *In vitro*, we demonstrated that

basolateral internalization of MBL by tubular cells induces ER-stress accompanied by excessive ATP production, loss of the mitochondrial membrane potential and production of mitochondrial superoxide followed by tubular cell death.

METHODS

Animals

The Animal Care and Use Committee of the Leiden University Medical Center approved all experiments. Eight-week-old male Lewis rats (200–250 gram) purchased from Harlan (Horst, The Netherlands), were housed in standard laboratory cages and were allowed free access to food and water throughout the experiments. Unilateral ischemia was induced by clamping of the left renal pedicle for 45 minutes using a bulldog clamp (Fine Science Tools, Heidelberg, Germany). During clamping the contralateral kidney was removed. Sham-treated rats had identical surgical procedures except for clamping of the left kidney but including removal of the contra-lateral kidney. Before induction of ischemia, rats were treated with anti-rat MBL-A mAb (IgG1; P7E4; 1 mg/kg) (11) or as a control anti-human fibronectin mAb (IgG1; 1 mg/kg). Rats were sacrificed 2, 5 or 24 h following reperfusion.

Assessment of kidney function

Renal function was determined by measuring creatinine and urea in serum samples using standard autoanalyzer methods by our hospital research services.

Renal histology

Histological evaluations were performed on formalin-fixed kidneys embedded in paraffin, which were sectioned and stained with silver by standard methods.

Renal immunostaining

Rat kidney sections (4 µm) of snap-frozen kidneys were air dried and acetone-fixed. Kidney sections were stained with digitonin (DIG)-conjugated mAb against CD43 (W3/13; macrophages), goat pAb against rat Kidney-injury molecule (KIM)-1 (R&D systems, Abingdon, UK) or rabbit pAb against rat GRP78 (Abcam), followed by horseradish peroxidase (HRP)-conjugated sheep anti-DIG (Roche Diagnostics, Mannheim, Germany), rabbit anti-goat (DAKO, Glostrup, Denmark) or goat anti-rabbit pAb (DAKO), respectively. The staining was visualized using Nova RED (Vector Labs, Peterborough, United Kingdom). Quantification of immunohistochemistry was performed in a blinded manner by assessing high power fields (HPFs; original magnification ×200) of the complete rat kidney using

a slide scanning microscope (Leica, Rijswijk, The Netherlands). Using imageJ software, the positive area in the outer medulla and cortico-medullary junction (expressed in pixels) was quantified.

RNA isolation and real-time PCR

RNA was extracted from snap frozen renal rat tissue or cultured human PTEC (HK-2) and purified using an RNeasy Mini isolation Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Specific rat primers for GRP78, CHOP and ribosomal protein S-15 (RPS-15, housekeeping gene), and specific human primers for spliced XBP-1 (12) and CHOP were designed using Oligo Explorer and obtained from Biolegio (Nijmegen, The Netherlands). The primer sequences were as follows: rat KIM-1 (forward, 5'-TACCAACATCAACCAGAGTCTC-3'; reverse, 5'-ACATAGAAGCCCTTAGTCGG-3'), rat CHOP (forward, 5'-GAGTCTAATACTCGATCATACC-3'; reverse, 5'-TTGATTCTTCCTCTTCGTTCC-3'), rat GRP78 (forward, 5'-GAGATTGTTCTGGTTGGCGGATCTACTC-3'; reverse, 5'-CCATATGCTACAGCCTCATCTGGGTT-3'), rat RPS-15 (housekeeping gene: forward 5'-CGTCACCCGTAATCCACC-3' and reverse 5'- CAGCTTCGCGTATGCCAC-3'), human CHOP (forward, 5'-GGAGCATCAGTCCCCACTT-3'; reverse, 5'-TGTGGGATTGAGGGTCACATC-3'), human sXBP-1 (forward, 5'-TGCTGAGTCCGCAGCAGGTG-3'; reverse, 5'-GCTGGCAGGCTCTGGGGAAG-3'), human IL-6 (forward, 5'- AAGCCAGAGCTGTGCAGATGAGTA-3'; reverse, 5'-AACAACAATCTGAGGTGCCCATGC-3') and human GAPDH (housekeeping gene: forward 5'-TTCCAGGAGCGAGATCCCT-3' and reverse 5'-CACCCATGACGAACATGGG-3'). PCR was performed using SYBR Green PCR mastermix (Biorad). Data were analyzed using Gene Expression Analysis for iCycler Real-Time PCR Detection System (Biorad). Expression of each gene was normalized against mRNA expression of the housekeeping gene rat RPS-15 or human GAPDH. RT PCRs were performed in duplicate.

XBP1 PCR splicing analysis

ER-stress-induced processing of rat XBP1 mRNA was evaluated by PCR and restriction site analysis. Primer for rat XBP-1, encompassing the IRE1 cleavage site of XBP1, was amplified using the rat XBP-1 primer (forward, 5'- AAACAGAGTAGCAGCGCAGACTGC-3'; reverse, 5'-GGATCTCTAAAACTAGAGGCTTGGTG-3') resulting in a 601-bp PCR product. PCR products were subsequently incubated with PstI at 37°C for 5 h followed by separation on 2% agarose gels. sXBP-1 lacks the PstI restriction site, which results in a larger PCR product. Upper bands representing spliced XBP-1 were quantified using image J software.

Cell culture

Immortalized human PTEC (HK-2) were grown as previously described (13). Cells were grown and exposed to MBL in 48-wells culture plates. To study basolateral exposure of MBL, cells were grown to 95% confluency in a transwell system (0,4 µM polyester membrane 12 mm inserts; Corning Life Sciences, New York, USA)

IL-6 ELISA

Secreted IL-6 in culture supernatants was measured using a commercial IL-6 sandwich ELISA (Sanquin, Amsterdam, Netherlands) according to the manufacturer's instructions.

Fluorescence microscopy

HK-2 cells were fixed using cold methanol or 1% paraformaldehyde (both from Pharmacy) and stained with mAb against human MBL (mAb 3E7; Hycult Biotech) and a rabbit pAb against human golgin-97 (Invitrogen, Carlsbad, USA), mitofilin (Abcam, Cambridge, UK) or GRP78 (Abcam) followed by alexa-488 conjugated goat anti-mouse IgG (Molecular Probes) and alexa-568 conjugated goat anti-rabbit IgG (Molecular Probes), respectively.

Mitochondrial assays

Mitochondrial membrane activity and mitochondrial superoxide production were assessed using MitoTracker Red and MitoSOX Red (both from Invitrogen), respectively. PTEC were exposed to MBL for consecutive time points followed by 15 minutes incubation with Mitotracker Red, MitoSOX Red was incubated during MBL exposure. Consequently, red fluorescence was assessed using fluorescence microscopy.

ATP measurement

Intracellular ATP was measured using a commericial ATP bioluminescent assay kit (Sigma Aldrich, Zwijndrecht, The Netherlands) according to the manufacturer's instructions.

Viability assays

HK-2 cells were washed and permeabilized using cold methanol and nuclei were stained using Hoechst. Viability was assessed by counting Hoechst-positive cells using fluorescence microscopy.

Preparation of human MBL

MBL was purified from human recalcified plasma as previously described (14;15).

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM) and subjected to nonparametric statistical analysis using one- and two-factor ANOVA or Mann-Whitney test using GraphPad Prism software. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Basolateral interaction with MBL is cytotoxic for human tubular cells

Recently, we demonstrated direct cytotoxicity of MBL towards tubular cells following renal I/R (11). In the present study we set out to dissect the mechanism by which MBL mediates tubular cell death. First, the *in vivo* condition following renal I/R was mimicked by exposing human PTEC (HK-2) to purified human MBL. Exposure to MBL at 4°C for 1 h resulted in extensive binding of MBL as assessed by fluorescence imaging and FACS analysis (Fig 1A). Incubation at 37°C, which allows internalization, revealed that MBL was internalized within minutes and trafficked to a perinuclear region (Fig 1B). Prolonged incubation with MBL resulted in extensive vacuolization after 8 h followed by rounding and detachment of cells within 24 h (Fig 1C). Vascular leakage following reperfusion results in exposure of tubular cells to MBL from the basolateral side, from where MBL should be subsequently internalized (11). To mimic this process in our *in vitro* model, we made use of a transwell system and exposed PTEC from the upper (apical) or lower (basolateral) compartment. Only basolateral exposure of PTEC to human MBL induced cell death, whereas apical exposure only had minimal effect (Fig 1D). This suggests that a basolateral membrane protein or internalization process is involved in this MBL-mediated cell death.

Internalized MBL colocalizes with the endoplasmic reticulum and mitochondria

Subsequently, intracellular trafficking of MBL was studied. No colocalization of MBL with the Golgi was found, whereas staining for GRP78 (Fig 2), an ER-residing protein, revealed a strong colocalization suggesting trafficking of MBL to the ER. GRP78 is a stress-inducible-ER chaperone, which serves as a master modulator of the ER-stress response. Recently, it was shown that GRP78 under stress conditions also traffics to mitochondria (16). Staining for MBL and a mitochondrial marker (mitofilin) indeed revealed a partial colocalization, suggesting that MBL might interacts with GRP78-positive mitochondria. Colocalization of MBL with GRP78 or mitofilin was subsequently confirmed by confocal microscopy.

MBL induces ER-stress in human tubular cells

GRP78 has been shown to prevent oxidative stress, calcium disturbances and cell death in PTEC and is indispensable for tubular cell homeostasis (17-19). To study the functional consequences of MBL internalization and interaction with GRP78, several ER-stress markers were assessed following exposure to MBL. Splicing of mRNA sXBP-1 by IRE1, expression of CHOP as a consequence of PERK and eIF2a phosphorylation, and IL-6 expression induced by JNK-pathway were assessed as markers for ER-stress and revealed a twenty- and tenfold induction of sXBP-1 (Fig 3A) and CHOP (Fig 3B) respectively. sXBP-1 and CHOP expression occurred within 30 minutes indicating that ER-stress mediated by MBL is one of

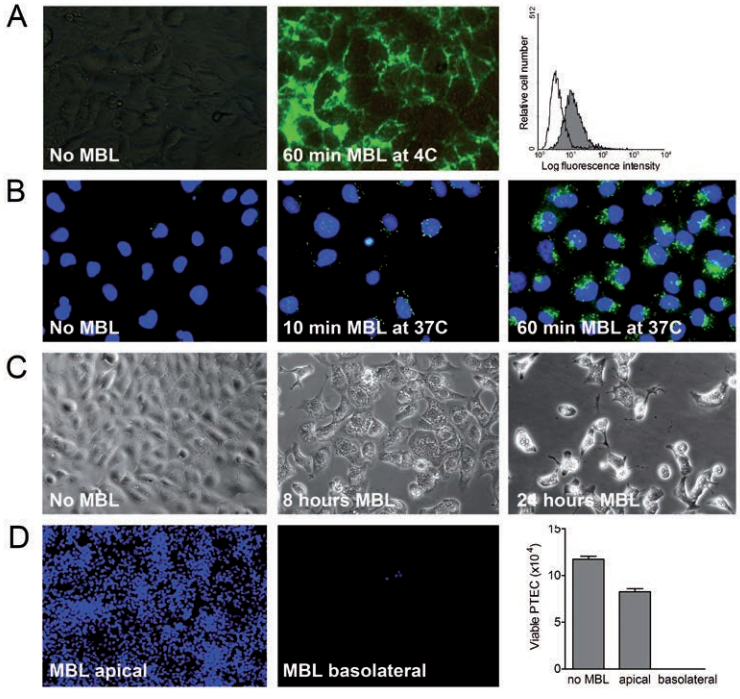

Figure 1. Basolateral interaction with MBL is cytotoxic for human tubular cells. Human PTEC (HK-2) were incubated with 10 µg/mL MBL for 1 h at 4°C (A) or 10 and 60 min at 37°C (B). Cells were stained extracellular (A) or intracellular (B) for MBL (green) and cells were photographed using a fluorescence microscope (original magnification ×200) or analysed by flow cytometry (A). Nuclei (blue) were stained using Hoechst (B). Cells were incubated with 10 µg/mL human MBL for 8 or 24 h at 37°C (C) and cells were photographed (original magnification ×200). To study apical or basolateral exposure to MBL (D), cells were cultured in a transwell system and exposed to 10 µg/mL MBL from the upper (apical) or lower (basolateral) compartment for 24 h. Cell viability was assessed by counting Hoechst-positive, but PI-negative cells in the well using fluorescence microscopy (original magnification ×100). Values shown are mean ± SEM of triplicate determinations from a single experiment and are representative of two similar transwell experiments.

the first consequences of MBL exposure. ER-stress following MBL exposure was accompanied by a strong and dose-dependent IL-6 protein expression (Fig 3C). Exposure to 10 or 20 µg/mL MBL induced a rapid cell death within 12 h, which might explain the lower levels of IL-6 in these conditions.

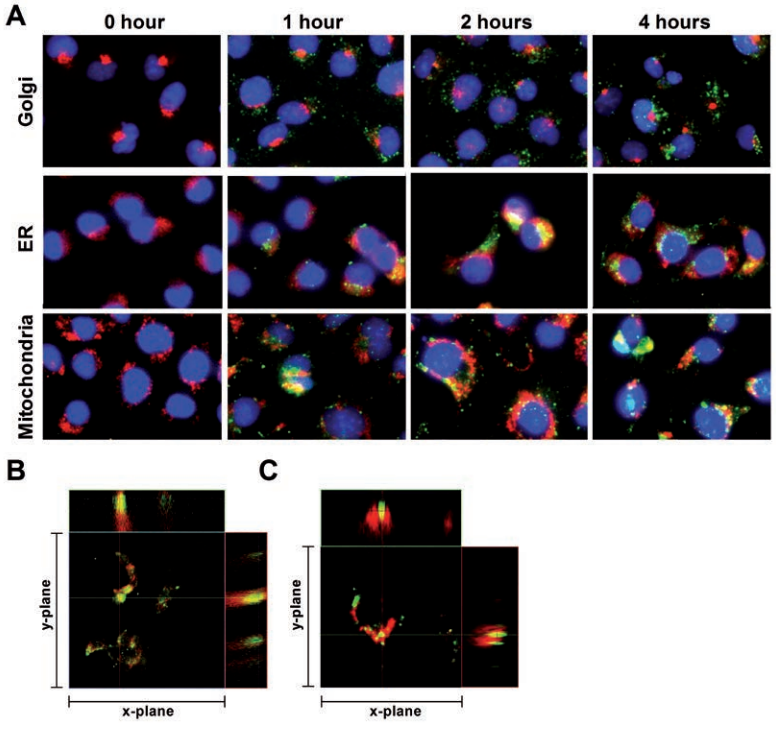

Figure 2. Intracellular trafficking of internalized human MBL. PTEC (HK-2) were exposed to MBL for 1, 2 or 4 h at 37°C, fixed and stained (A) for MBL (green) and a marker (red) for the golgi (golgin), endoplasmic reticulum (GRP78) or mitochondria (mitofilin). Nuclei (blue) were stained using Hoechst. Double staining (yellow) was found for MBL with GRP78 or mitochondria, but not with golgin. Cells were photographed using fluorescence microscopy (original magnification x600). PTEC (HK-2) were exposed to MBL for 4 h at 37°C, fixed and stained for MBL (green) and a marker (red) for the endoplasmic reticulum (B, GRP78) or mitochondria (C, mitofilin). Nuclei (blue) were stained using Hoechst. Colocalization (yellow) was assessed by confocal microscopy.

MBL affects mitochondrial homeostasis

ER-stress results in calcium flux from the ER into the cytosol, which is buffered to some degree by mitochondrial calcium uptake. Increased mitochondrial calcium stimulates oxidative metabolism and ATP production. This prompted us to study whether MBL-mediated ER-stress might influence mitochondrial homeostasis. Assessment of cellular ATP levels revealed that ER-stress was followed by an

excessive ATP production in the first hours of MBL exposure. Ablation of ATP production at 24 h was accompanied by tubular cell death. (Fig 4A).

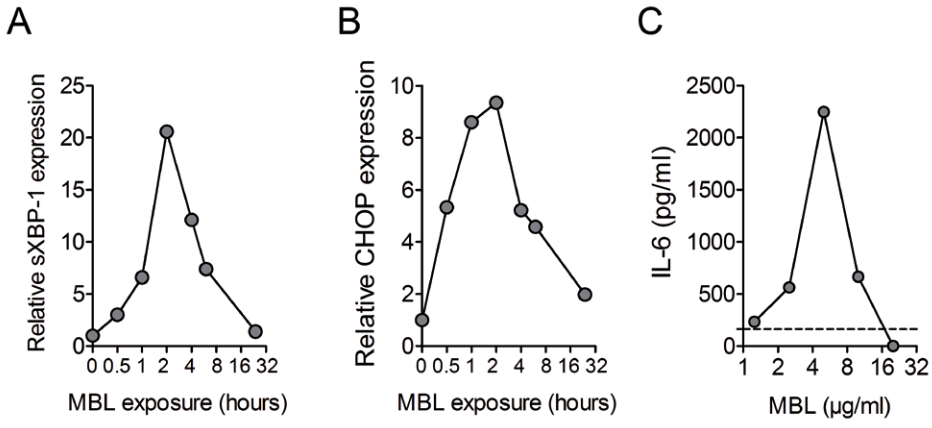

Figure 3. MBL induces ER-stress in human PTEC. PTEC (HK-2) were incubated with 10 µg/mL MBL for consecutive time points. Subsequently, mRNA was harvested and expression of spliced (s)XBP1 (A) and CHOP (B) was assessed by RT-PCR. PTEC were incubated with MBL (0-10 µg/mL) for 24 h. Culture supernatants were collected and secreted IL-6 content was measured by an IL-6 sandwich ELISA (C).

Prolonged ER-stress might lead to mitochondrial injury induced by excessive calcium influx into the mitochondria. Strong calcium entry activates mitochondrial superoxide generation and collapse of the mitochondrial membrane potential. In addition, mitochondrial GRP78 has been shown to stabilize Raf-1 to maintain mitochondrial permeability and to protect cells from ER-stress-induced apoptosis (20). This prompted us to more in-depth study mitochondrial homeostasis.

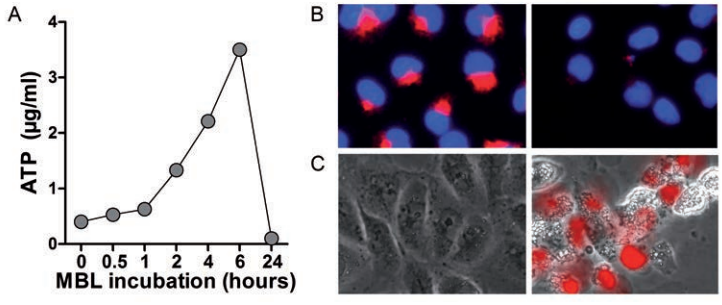

Figure 4. MBL affects mitochondrial homeostasis. PTEC (HK-2) were exposed to 10 µg/mL MBL for 0-24 h, after which intracellular ATP content was measured (A). PTEC (HK-2) were exposed to 10 µg/mL MBL for 4 h (B, C). To assess the mitochondrial membrane potential (B), cells were incubated with MitoTracker Red for 15 min after MBL exposure. Cells were fixed and nuclei (blue) were stained using Hoechst. To assess superoxide production (C), cells were incubated with MitoSOX Red during 4h MBL exposure. Cells were photographed using a fluorescence microscope (original magnification x600).

Active mitochondria were stained using MitoTracker Red, which accumulation in mitochondria is dependent on the mitochondrial membrane potential. Normal PTEC showed active mitochondria, however exposure to MBL for 4 h dramatically reduced the mitochondrial membrane potential (Fig 4B). Loss of membrane potential was accompanied by mitochondrial superoxide production, as assessed by MitoSOX Red which specifically is targeted to active mitochondria and upon oxidation by mitochondrial superoxide produces red fluorescence (Fig 4C). The massive increase in ATP production, loss of mitochondrial membrane potential and ROS formation were preceded by a period of extensive ER-stress (Fig 3), suggesting that mitochondrial alterations are the result of the initial ER-stress induced by MBL.

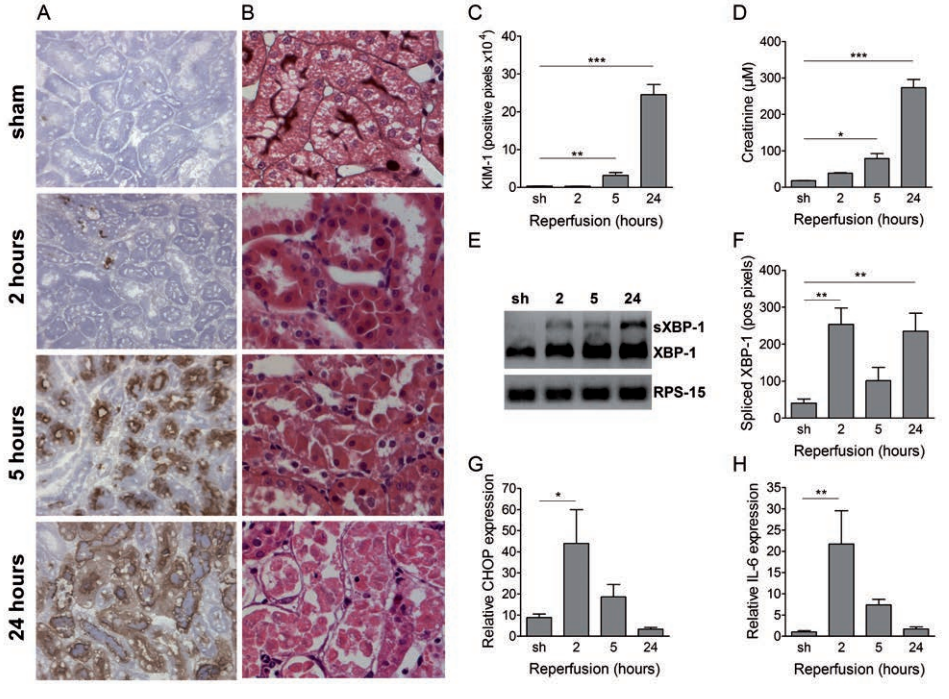

Figure 5. Renal I/R is accompanied by ER-stress and tubular injury. Lewis rats (n=6 per group) underwent unilateral ischemia for 45 min and removal of the contralateral kidney. Rats (n=6 per group) were sacrificed at consecutive time points and renal sections were stained for Kidney-injury molecule (KIM)-1 (A, original magnification x200) or processed for silver staining (B, original magnification x400). KIM-1 was quantified using digital image analysis (C). Following reperfusion or in sham (sh) operated rats, blood samples were drawn at consecutive time points to assess renal function by measuring serum creatinine (D). Renal mRNA expression of rat spliced (s)XBP-1 (E,F). CHOP (G) and IL-6 (H) was assessed in renal tissue at consecutive time points following reperfusion or in sham (sh) operated rats. Data are shown as mean ± SEM, n = 6 per group. The comparison between groups is indicated by asterisks. *p<0.05; **p<0.01; ***p<0.001.

Renal I/R is accompanied by ER-stress and tubular injury

To study the *in vivo* relevance of these findings, we assessed the role of MBL in ER-stress in a rat model of renal IRI. Firstly, tubular injury and occurrence of ER-stress was assessed. Clamping of the renal pedicle for 45 minutes and removal of the contralateral kidney resulted in severe renal dysfunction (Fig 5D) within 24 h, which was accompanied by extensive protein expression of Kidney-injury molecule (KIM)-1 within 5 h upon reperfusion (Fig 5A, C). A silver staining revealed that tubular alterations and loss of tight junctions already occurred within 2 h upon reperfusion (Fig 5B). Interestingly, this injury was accompanied by a significant splicing of XBP-1 (Fig 5E, F), expression of CHOP (Fig 5G) and IL-6 (Fig 5H) 2 h after reperfusion.

Renal I/R is accompanied by loss of ER-stress sensor GRP78

Staining for KIM-1 revealed that mainly the tubuli in the corti-medullary transition were injured and extensively expressed KIM-1 at 24 h after reperfusion (Fig 6A). Strikingly, staining for GRP78 revealed that particularly in this region, tubular GRP78 protein expression was lost (Fig 6B) already 2 h after reperfusion. While some tubuli normally expressed GRP78, others completely lost expression (Fig

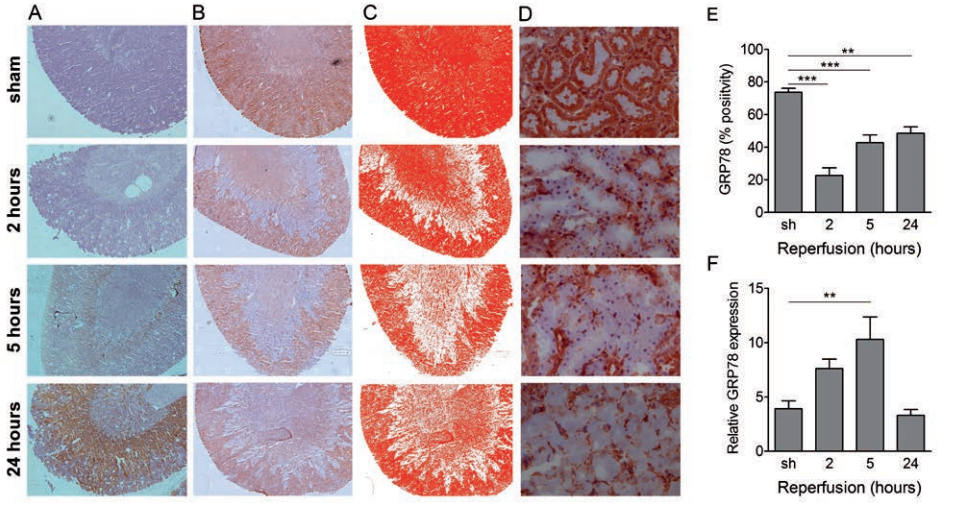

Figure 6. Renal I/R is accompanied by loss of ER-stress sensor GRP78 in the cortico-medullary tubular cells. Renal sections (n=6 per group) from sham operated rats or rats sacrificed 2, 5 or 24 h after reperfusion were stained for KIM-1 (A) or GRP78 (B). Sections were photographed and photographs were digitally stitched and analyzed (C) using digital image. GRP78 staining in the cortico-medullary region (D, original magnification x400) was digitally quantified (E). GRP78 mRNA expression (F) was assessed in total renal tissue at consecutive time points following reperfusion or in sham (sh) operated rats. Data are shown as mean ± SEM, n = 6 per group. The comparison between groups is indicated by asterisks. *p<0.05; **p<0.01; ***p<0.001.

6D), which might result in extensive ER-stress in these cells. Quantification (Fig 6C, E) showed that most staining was significantly lost within 2 h upon reperfusion which was accompanied by an increased mRNA expression of GRP78.

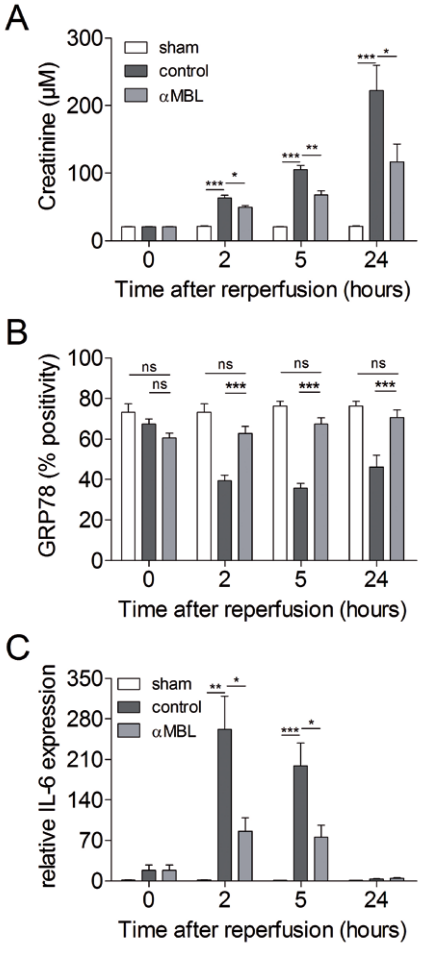

Figure 7. Inhibition of MBL prevents loss of ER-stress sensor GRP78 and subsequent IL-6 expression. Before induction of 45 min of unilateral ischemia and removal of the contralateral kidney, rats received anti-MBL-A or an isotype-matched control mAb (1 mg/kg). Before and following reperfusion blood samples were drawn at consecutive time points to assess renal function by measuring serum creatinine (A) Renal sections (n=6 per group) from sham operated, control-treated or anti-MBL treated rats sacrificed 2, 5 or 24 h after reperfusion were stained for GRP78. Staining in the cortico-medullary region was subsequently digitally quantified (B). IL-6 mRNA expression (C) was assessed in renal tissue at consecutive time points. Data are shown as mean ± SEM, n = 6 per group. The comparison between groups is indicated by asterisks. *p<0.05; **p<0.01; ***p<0.001.

Inhibition of MBL prevents loss of ER-stress sensor GRP78

To assess the role of MBL in this, we therapeutically target MBL using the protective and blocking mAb (P7E4) to MBL (11). Inhibition of MBL following I/R significantly protected against renal dysfunction (Fig 7A). Importantly, inhibition of MBL completely prevented the loss of tubular GRP78 early after reperfusion, whereas control-treated animals lost their GRP78 protein expression in the cortico-medullary tubular cells (Fig 7B). Loss of GRP78 was accompanied by an extensive expression of IL-6, which might be related to ER-stress and loss of GRP78. In addition, anti-MBL treatment also significantly reduced IL-6 expression

early after reperfusion (Fig 7D), suggesting that MBL plays an important role in early tubular ER-stress following reperfusion. These findings await further studies on the role of MBL in tubular mitochondrial homeostasis *in vivo* which might be closely related to this process.

DISCUSSION

Recently we demonstrated a pivotal role for MBL in the pathogenesis of renal IRI (11). MBL has cytotoxic effects towards PTEC independent of complement activation contributing to tubular injury following reperfusion. Here, we demonstrate that MBL interacts with GRP78 in PTEC, mediates ER-stress and affects mitochondrial homeostasis. Therapeutic inhibition of MBL *in vivo* protected tubular cells from loss of vital GRP78 and consequent tubular injury

GRP78 is a constitutively expressed molecular chaperone, however expression is enhanced under a variety of stressful conditions including hypoxia, glucose deprivation, alterations in intracellular calcium and oxidative stress (21), events that take place during renal I/R. Induction of GRP78 is critical for maintaining viability of cells that are subjected to such stresses and loss of GRP78 would therefore be detrimental. Here, we show that renal I/R is accompanied by ER-stress and loss of GRP78 in the cortico-medullary region of kidney, the region that is mostly affected. Importantly, therapeutic inhibition of MBL protected tubular cells located in this region from loss of vital GRP78 and consequent tubular injury, indicating that MBL might be injurious to tubular cells by interfering with GRP78. Expression of proinflammatory IL-6, a consequence of GRP78 degradation (22) and ER-stress (23;24), was also significantly reduced by inhibition of MBL following reperfusion.

GRP78 has been shown to prevent oxidative stress, calcium disturbances and cell death in renal epithelial cells (17;25). Expression of antisense RNA, targeted to GRP78 sensitized tubular cells to oxidant-induced cell death (19), indicating that GRP78 is indispensable in tubular cells under oxidative stress.

Internalization of circulation-derived MBL *in vivo* was shown to be the process by which tubular epithelial cells following renal I/R might be injured (11). By exposing tubular cells *in vitro* to MBL, we here demonstrated that basolateral internalization of MBL by tubular cells induced a potent ER-stress response which was accompanied by an excessive ATP production, a subsequent loss of the mitochondrial membrane potential, production of mitochondrial superoxide followed by induction of cell death. Internalized MBL colocalized with mitochondria and GRP78. Although GRP78 is mostly known from its function as molecular chaperone in the ER, GRP78 has also been shown to be

present, although in low amounts, on mitochondria, in the cytoplasm, on the cell membrane and as a secreted form (16;26-30). Membrane-bound GRP78 might be a possible candidate as a cellular receptor by which MBL is internalized. Indeed, it has been shown that MBL is able to interact with GRP78 in hepatic cells (31), suggesting that a detrimental interaction with GRP78 in tubular cells might be possible, however the mechanism by which MBL is internalized is still subject of investigation. Interestingly, overexpression of calreticulin, another ER chaperone also protected tubular cells against oxidative stress (18). Calreticulin has been described as the MBL receptor on phagocytosing cells (32;33), making this also a possible candidate by which MBL might be internalized.

The mechanism by which MBL affects mitochondrial homeostasis and ER-stress is still speculative since both processes are tightly intertwined. Depletion of cellular ATP as a consequence of ischemia or oxidative stress is known to induce ER-stress, since most GRP78 uses the energy from ATP hydrolysis to promote folding and prevent aggregation of proteins within the ER (34). Vice versa, ER-stress enhances mitochondrial calcium to generate more ATP (35;36). However, excessive calcium flux from the ER to the mitochondria during prolonged ER-stress will lead to mitochondrial injury and subsequent ATP depletion (7;37). GRP78 has been shown to be involved in both processes. First of all, GRP78 prevents calcium flux from the ER (36), probably by interacting with the translocon calcium channel in the ER-membrane (8). In addition, GRP78 on mitochondria has been shown to stabilize the mitochondrial membrane potential by associating with raf-1 and cooperatively confer resistance to ER-stress induced cell death (20). Interference of MBL with GRP78 might affect both processes. Our data suggest that initially ER-stress is induced following MBL exposure, followed by disturbances in mitochondrial homeostasis. Release of calcium from ER stores might be the main cause of MBL-mediated ER-stress. An increase in cytosolic calcium in tubular cells then would lead to enhanced calcium influx into mitochondria, disrupting mitochondrial metabolism eventually leading to cell death.

Recently is has been shown that ligation of cell-surface GRP78 on cancer cells also causes release of calcium from ER stores mediated via phospholipase C, thereby increasing cytosolic calcium (38). In line with this, it is known that especially tumor cells have increased levels of GRP78 on the cell surface. Importantly, is has been shown that MBL bears antitumor effects, which has been termed MBL-dependent cell-mediated cytotoxicity (39). Since MBL is able to interact with GRP78, we speculate that, next to killing of tumor cells, the cytotoxic effect of MBL on tubular cells might also be mediated by signaling through cell surface GRP78.

Under physiologic conditions GRP78 is hardly present on normal cells, however

induction of ER-stress has been shown to promote GRP78 localization on the surface of kidney cells (30). ER-stress induced by glucose deprivation during renal ischemia might be the initial trigger for GRP78 to traffic to the basolateral membrane of the tubular cells. Especially tubular cells in the cortico-medullary border are subjected to low oxygen and glucose levels during I/R, making these cells particular sensitive to ER-stress. It is therefore tempting to speculate that vascular leakage following reperfusion enables circulation-derived MBL to enter the interstium and bind to GRP78 on the cell surface of the tubular cells. Signaling to GRP78 might then have detrimental effects. Next to signaling, internalization of MBL might also be mediated by GRP78, since evidence has emerged that GRP78 also serves as a receptor for viral entry into host cells (40).

In this study we show that loss of GRP78 *in vivo* is tightly connected to MBL, because inhibition of MBL completely preserved GRP78 protein expression in the cortico-medullary tubular cells. However, the mechanism by which GRP78 is degraded is still unknown. Recently, it was shown in macrophages that the mycotoxin deoxynivalenol induced ER-stress, which was accompanied by loss of GRP78 and high expression of IL-6(22). The authors suggested that the loss of GRP78 was related to an autophagic pathway. The process of autopahgy has also been described in renal IRI and might be involved in degradation of GRP78. In addition, it has been shown that cytosolic GRP78 might be secreted(26), however the mechanism involved requires further investigation.

In summary, our results document the important role of MBL in renal IRI. We demonstrated that inhibition of MBL *in vivo* protected tubular cells from loss of vital GRP78 and consequent tubular injury. Basolateral internalization of MBL by tubular cells *in vitro* induced ER-stress accompanied by excessive ATP production, loss of mitochondrial membrane potential and production of mitochondrial superoxide followed by tubular cell death. These results indicate that therapeutic intervention at the level of MBL might be a promising target in kidney transplantation.

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CHAPTER 7

IN PREPARATION

Recombinant human C1 inhibitor fails to reduce Mannan-binding lectin-mediated tubular injury in a rat model of renal ischemia/reperfusion

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ABSTRACT

Ischemia/reperfusion injury (IRI) is a key event in kidney transplantation and is accompanied by activation of the complement cascade. In addition, a novel and complement-independent role for Mannan-binding lectin (MBL), initiator of the lectin pathway of complement, was established in the pathogenesis of renal IRI. Recombinant human C1-inhibitor (rhC1INH), a serine protease inhibitor, inhibits complement activation and through its differential glycosylation might also directly interact with MBL. Therefore, application of rhC1INH might be a potential therapeutic option to prevent or reduce renal IRI.

Here, we explored the therapeutic application of rhC1INH in a rat model of renal IRI and studied whether rhC1INH is able to attenuate MBL-mediated tubular injury. We demonstrate that rhC1INH was functionally active in rat serum and blocks both classical and lectin pathway activation *in vitro*. However, the therapeutic administration of rhC1INH *in vivo* did not attenuate renal dysfunction, tubular injury, inflammatory cell influx and complement cascade activation following reperfusion in rats. Kinetic studies revealed that rhC1INH was mostly cleared from the circulation within 2 hours, complement activity was only briefly reduced and circulating MBL levels were unaffected. In addition, we demonstrated that rhC1INH did not block the MBL-mediated cytotoxicity towards human tubular cells *in vitro*.

Taken together, we demonstrate that rhC1INH is not protective in a rat model of renal IRI and does not rescue human tubular cells from MBL-mediated cytotoxicity *in vitro*.

INTRODUCTION

Ischemia caused by inadequate local blood supply, is an inevitable event in kidney transplantation. Restoration of blood flow to ischemic tissue during the transplantation procedure paradoxically exacerbates tissue damage by initiating a cascade of inflammatory events including endothelial dysfunction, formation of reactive oxygen species, neutrophil sequestration and complement activation, which all contribute to post-ischemic injury (1-3).

Numerous clinical and experimental studies have shown that renal IRI has a major impact on short- and long-term graft survival after kidney transplantation and usually accounts for delayed graft function and associated morbidity and mortality in the clinic (3-5). Complement activation is a key feature of renal ischemia/reperfusion injury (IRI) and has been demonstrated both in the clinical setting (6;7) as well as in experimental models (8-10).

The complement system is a complex cascade of around thirty plasma proteins that can be activated via either the classical pathway (CP), lectin pathway (LP) or alternative pathway (AP)(11). The CP is activated by binding of C1q to e.g. antigen-antibody complexes or apoptotic cells (12) which lead to activation of the associated serine proteases C1r and C1s. The LP is activated by binding of Mannan-binding lectin (MBL) or ficolins to sugar moieties which lead to activation of the MBL-associated serine proteases (MASPs). Activated serine proteases C1r/C1s or MASPs then cleave C4 and C2, which result in the generation of the classical C3 convertase. The alternative pathway (AP) is continuously activated at a low level (so-called tickover), does not require C4 and is tightly regulated by membrane-bound and soluble complement regulators. Furthermore, properdin can act as a focal point of AP-mediated complement activation upon binding to its ligand (13). All three pathways converge at the level of complement factor C3, which is cleaved and subsequently deposits as C3b on target cells resulting in opsonization and clearance by phagocytic cells. Further activation of the complement cascade leads to generation of the membrane attack complex (MAC) C5b-9 and lysis of target cells.

C1 esterase inhibitor (C1INH), a physiologic serine protease inhibitor belonging to the serpin superfamily, acts as a major inhibitor of complement system activation and prevents uncontrolled activation (14-16). It is an acute phase protein with a mean plasma level of 250 µg/ml, and is increasing up to 2.5-fold during inflammation. 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) (17) and C1rC1s(C1INH)2 complexes (18-20), which subsequently dissociate from the MBL or C1q molecule, respectively. These complexes are then rapidly cleared from the circulation.

Renal IRI is associated with extensive loss of the cortico-medullary proximal tubular epithelial cells (PTEC), an event often referred to as acute tubular necrosis (ATN). Recently, we demonstrated that therapeutic inhibition of MBL with a monoclonal antibody was protective against renal IRI and more in-depth studies revealed a direct cytotoxic effect of MBL on tubular epithelial cells independent of complement activation (18). Upon reperfusion of the ischemic kidney, MBL from circulation leaks into the interstitium where it is internalized by tubular cells and contributes to tubular injury. Unfortunately, to date no anti-MBL therapy or other treatments for renal IRI in the clinic exists.

Recently however, several clinical possibilities of pharmacologic inhibition of complement, including C1INH have become available (21). C1INH might be a possible therapeutic candidate to prevent renal IRI given that C1INH regulates vascular permeability (22) and might prevent leakage of MBL in the interstitial compartment following reperfusion. Secondly, C1INH targets the MASPs,

which might be involved in the cytotoxic effect of MBL on tubular cells. Thirdly, complement that is activated at a later stage following reperfusion leading to exacerbation of tissue injury, might be inhibited.
Recombinant human C1INH (Ruconest®), which recently has been registered for treatment of acute attacks in hereditary angioedema, is differentially glycosylated (23;24) with increased oligo-mannose structures and therefore, compared to plasma-derived C1INH, might include an additional inhibitory effect towards MBL recognizing mannose residues.
In the present study, we explored the therapeutic application of rhC1INH (Ruconest®) in a rat model of renal IRI and studied whether rhC1INH is able to attenuate MBL-mediated tubular injury. We demonstrate that rhC1INH is not protective in a rat model of renal IRI and does not rescue human tubular cells from MBL-mediated cytotoxicity *in vitro*.

METHODS

Animals

The Animal Care and Use Committee of the Leiden University Medical Center approved all experiments. Eight-week-old male Lewis rats (200–250 gram) purchased from Harlan (Horst, The Netherlands), were housed in standard laboratory cages and were allowed free access to food and water throughout the experiments. Unilateral ischemia was induced by clamping of the left renal pedicle for 45 minutes using a bulldog clamp (Fine Science Tools, Heidelberg, Germany). During clamping the contralateral kidney was removed. Sham-treated rats had identical surgical procedures except for clamping of the left kidney but including removal of the contra-lateral kidney. Before induction of ischemia, rhC1INH (Ruconest®, 625 U/kg, equivalent to 100 mg/kg; Pharming Group NV, Leiden, The Netherlands) or as a control human serum albumin (HSA; 100 mg/kg) was infused intravenously. Blood samples were collected at 1, 2, 5 or 24 hours following reperfusion and processed as serum. Kidneys were harvested 24 hours after reperfusion and subsequently animals were sacrificed.

Assessment of kidney function

Renal function was determined by measuring creatinine and urea in serum samples using standard autoanalyzer methods by our hospital research services.

Renal immunostaining

Rat kidney sections (4 μm) of snap-frozen kidneys were air dried and acetone-fixed. Kidney sections were stained with digitonin (DIG)-conjugated mAb

against CD68 (ED-1, macrophages) or goat pAb against rat KIM-1 (R&D systems, Abingdon, UK), followed by horseradish peroxidase (HRP)-conjugated sheep anti-DIG (Roche Diagnostics, Mannheim, Germany) or rabbit anti-goat (DAKO, Glostrup, Denmark), respectively. The staining was visualized using Nova RED (Vector Labs, Peterborough, United Kingdom). Rat C3 deposition was assessed by direct staining using a FITC-conjugated rabbit polyclonal to rat C3 (made in-house). Quantification of immunohistochemistry was performed in a blinded manner by assessing 10 consecutive high power fields (HPFs; magnification ×200) of the outer medulla and cortico-medullary junction on each section. Using image J software, the positive area (expressed in pixels) was quantified.

Rat serum complement activity measurements

Functional activity of the lectin and classical pathway were assessed using plates coated with mannan or IgM, as previously described (25), with minor modifications. Rat sera drawn at different time points after reperfusion or normal rat serum mixed with rhC1INH (Ruconest®, Pharming Group NV) were 1/50 diluted in GVB++ (veronal buffered saline, 0.1% gelatin, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, 0.05% Tween 20) and incubated for 60 min at 37°C to allow C3 deposition in the well. The plates were washed with PBS/0.05%Tween and incubated with dig-conjugated mouse anti-rat C3 antibody (ED11, made in-house (26)), followed by HRP-conjugated sheep anti-DIG (Roche Diagnostics). After washing, C3 deposition was quantified with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; from Sigma-Aldrich; 2.5 mg/ml in 0.1M Citrate/Na_2HPO_4 buffer, pH 4.2) in the presence of 0.01% H_2O_2, for 30–60 min at room temperature. The OD at 415 nm was measured using a microtiter plate reader (Biorad).

Rat MBL ELISA

MBL levels in rat serum were assessed using a sandwich ELISA. In short, Nunc Maxisorb plates (Nunc, Roskilde, Denmark) were coated with a mAb against rat MBL (P7E4, kindly provided by G.L. Stahl Harvard Medical, School, Boston, USA) followed by rat serum incubation. Rat MBL binding was detected using DIG-conjugated mAb against rat MBL (P7E4). After washing, MBL binding was quantified with ABTS; (Sigma-Aldrich). The OD at 415 nm was measured using a microtiter plate reader (Biorad).

MBL-MASP complex ELISA

Human MBL-MASP complexes after MBL purification were assessed using a sandwich ELISA. In short, Nunc Maxisorb plates were coated with a pAb against MASP-2 (kindly provided by R. B. Sim, University of Oxford, Oxford, U.K.) followed by sample incubation. MBL-MASP complex binding was detected

using DIG-conjugated mAb against human MBL (3E7, Hycult Biotecht, Uden, Netherlands). After washing, MBL-MASP complex binding was quantified with ABTS; (Sigma-Aldrich). The OD at 415 nm was measured using a microtiter plate reader (Biorad).

RhC1INH ELISA

Circulating levels of rhC1INH before, 1, 2, 5, and 24 hours after reperfusion were assessed using a sandwich ELISA as previously described (24).

Cell culture

Immortalized human PTEC (HK-2) were grown as previously described (27).

Fluorescence microscopy

HK-2 cells were fixed using cold methanol and stained with mAb against human MBL (mAb 3E7; Hycult Biotech) followed by Alexa-488 conjugated goat anti-mouse IgG (Molecular Probes). Nuclei were stained using Hoechst (Molecular Probes, Leiden, The Netherlands).

Viability assays

Cell viability was assessed using propidium iodide (PI; Molecular Probes, Leiden, The Netherlands). Following incubation with PI, cells were washed and permeabilized using cold methanol and nuclei were stained using Hoechst. Viable cell count was assessed by counting Hoechst-positive, but PI-negative cells using fluorescence microscopy.

Preparation of human MBL and MASP-free MBL

MBL was purified from human recalcified plasma as previously described (28). To yield a MASP-free MBL preparation, MBL was further purified by gel filtration using a Sepharose 6B column as previously described (29). In short, the purified MBL preparation was dialysed against 0.1 M acetic-acid containing 0.2 M NaCl and 5 mM EDTA at pH 5. Subsequently, the dialysed sample was subjected to size exclusion chromatography on a Superdex 200. This procedure results in dissociation of purified MBL–MASP complexes which were subsequently separated by size exclusion.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM) and subjected to nonparametric statistical analysis using one- and two-factor ANOVA or Mann-Whitney test using GraphPad Prism software. A value of $P < 0.05$ was considered statistically significant.

RESULTS

RhC1INH is functionally active in rat serum

Before therapeutic application rhC1INH (Ruconest®) in a rat model of renal IRI, functionality of rhC1INH in rat serum was tested *in vitro*, using a complement activation assay assessing classical and lectin pathway activity. Preincubation of rat serum with rhC1INH resulted in a dose-dependent inhibition of C3 deposition (Fig 1). The lectin pathway was more effectively inhibited by rhC1INH than the classical pathway (Fig 1B), with an IC50 of 1 and 5 U/ml, respectively. Maximum inhibition of lectin pathway activation was achieved at 10 U/ml. Therefore, a therapeutic dose of 625 U/kg was chosen for *in vivo* application in rats, corresponding to an estimated end concentration of at least 10 U/ml rhC1INH in circulation.

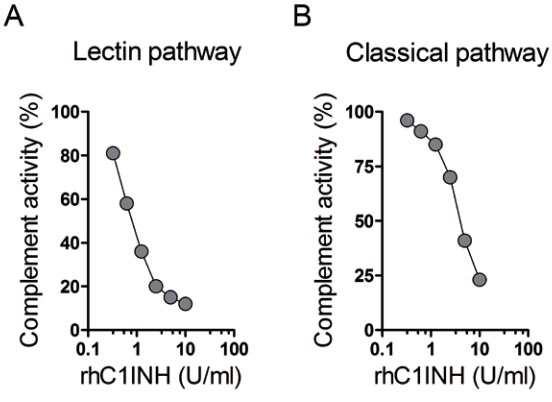

Figure 1. RhC1INH inhibits complement activation in rat serum. Preincubation of rat serum with rhC1INH resulted in a dose-dependent inhibition of C3 deposition on mannan- (A) and IgM-coated plates (B). C3 deposition as a measure of complement activity was calculated as a percentage of C3 deposition in wells with untreated rat serum.

RhC1INH does not preserve renal function after ischemia/reperfusion

Five minutes before induction of renal ischemia, rats (n=6) were infused with rhC1INH (625 U/kg) or as a control human serum albumin (HSA). Induction of ischemia for 45 min followed by reperfusion resulted in a significant renal dysfunction in control-treated rats as measured by serum levels of creatinine (Figure 2A) and urea (Figure 2B). Treatment with rhC1INH did not protect against renal dysfunction. Rats treated with rhC1INH even tended to show a higher increase in creatinine and urea (Fig 2B) compared to control treated animals at 24 hours of reperfusion.

RhC1INH does not reduce C3 deposition, cellular influx and tubular injury

Rats were sacrificed at 24 hours of reperfusion and deposition of C3 was assessed. While kidneys from sham-operated rats showed a C3 staining with a typical half-moon distribution surrounding tubular cells, following IRI there

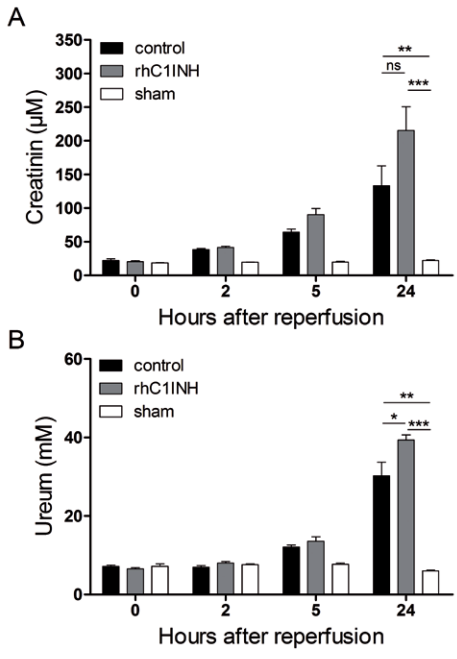

Figure 2. RhC1INH does not preserve renal function after ischemia/ reperfusion. Before induction of unilateral ischemia and removal of the contralateral kidney, rats received rhC1INH (625 U/kg) or as a control human serum albumin (100 mg/kg). Before and following reperfusion, blood samples were drawn at consecutive time points to assess renal function by measuring serum creatinine (A) and urea (B) levels. Data are shown as mean ± SEM, n = 6 per group. The comparison between groups is indicated by asterisks. *p<0.05; **p<0.01; ***p<0.001.

was a significant increase in tubular C3 deposition (Fig 3A). Although rhC1INH was able to inhibit C3 deposition *in vitro* (Fig 1), administration *in vivo* did not reduce tubular deposition of C3. Also influx of macrophages (Fig 3B), which was apparent in the control treated animals, was not reduced in the rhC1INH-treated group. Expression of kidney injury molecule (KIM)-1, an early marker for tubular injury, was strongly expressed at 24 hours following reperfusion (Fig 3C) and was also not reduced by application of rhC1INH.

Kinetics of rhC1INH in rats are unfavorable for effective protection

Assessment of complement activity in circulation revealed that 1 hour following reperfusion both the lectin and classical pathway were blocked efficiently by rhC1INH. However, this inhibitory effect was only short, since at 2 hours of reperfusion, complement activity of both lectin (Fig 4A) and classical (Fig 4B) pathway was restored to basal levels. This prompted us to study the pharmacokinetics of rhC1INH *in vivo*. Measurement of rhC1INH levels following administration revealed a fast clearance of rhC1INH from circulation. One hour after reperfusion still sufficient rhC1INH levels (Fig 4C) for lectin and classical pathway inhibition in circulation (Fig 4A, B) were detected (10 U/ml) as was also calculated from the assay assessing rhC1INH functionality in rat serum *in vitro* (Fig 1). In contrast, rhC1INH levels at 2 hours were reduced by more than threefold and 5 hours after reperfusion almost undetectable. From this, a half-life of approximately 30 minutes could be calculated. The data suggest that the

rhC1INH concentrations at 2 and 5 hours of reperfusion were not sufficient for effective complement inhibition in the circulation.

RhC1INH does not inhibit MBL-mediated tubular cell death

Recently we showed that inhibition of MBL by a monoclonal antibody is protective against renal IRI in rats (30). In addition, we showed that MBL directly induces tubular injury independent of complement activation. Assessment of circulating MBL levels after rhC1INH administration demonstrated that rhC1INH did not affect the levels of MBL in circulation (Fig 4D). Lectin pathway activity at 1 hour reperfusion was efficiently blocked, indicating that rhC1INH only targets and dissociated the MASPs from MBL, but did not affect MBL itself. To assess whether rhC1INH was able to prevent MBL-mediated tubular injury, human tubular epithelial cells were incubated with purified MBL *in vitro* (Fig 5A), which resulted in an extensive induction of cell death within 24 hours. Preincubation of MBL with a dose response of rhC1INH (10-1000 µg/ml, Fig 5A, B), however, did not protect tubular cells from MBL-mediated cell death.

RhC1INH is a protease inhibitor and irreversibly binds to and inactivates MBL-associated serine proteases (MASP)-1 and -2. To study whether these MASPs,

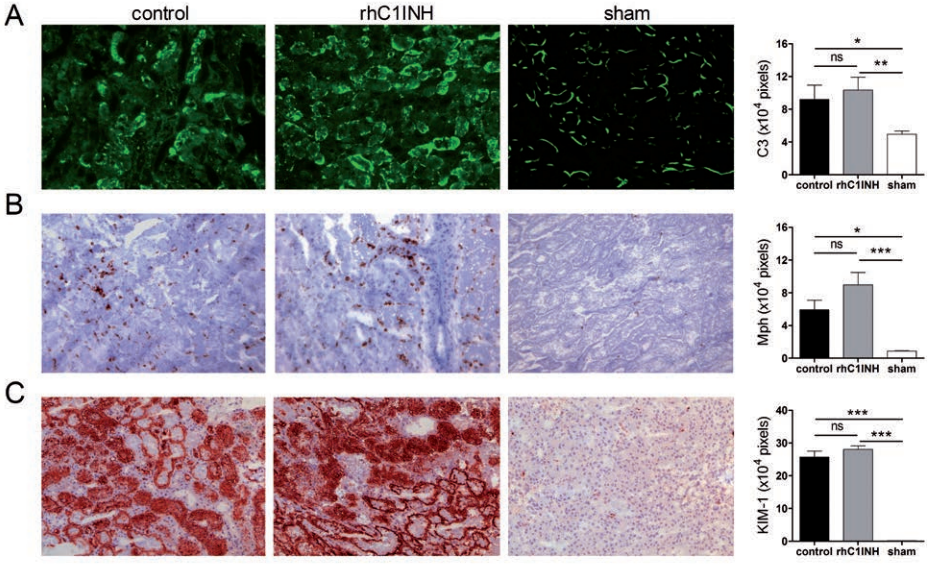

Figure 3. RhC1INH does not reduce C3 deposition, cellular influx and tubular injury. Renal sections from control- (A), rhC1INH-treated (B) or sham-operated rats 24 h following reperfusion were stained for C3 deposition (A) macrophages (Mph; B) or kidney-injury molecule (KIM)-1 (C). C3 deposition, Mph infiltrate and KIM-1 expression were quantified using digital image analysis. Data are shown as mean ± SEM (n = 6 per group). Original magnification ×200. The comparison between groups is indicated by asterisks. *p < 0.05; **p<0.01; ***p<0.001.

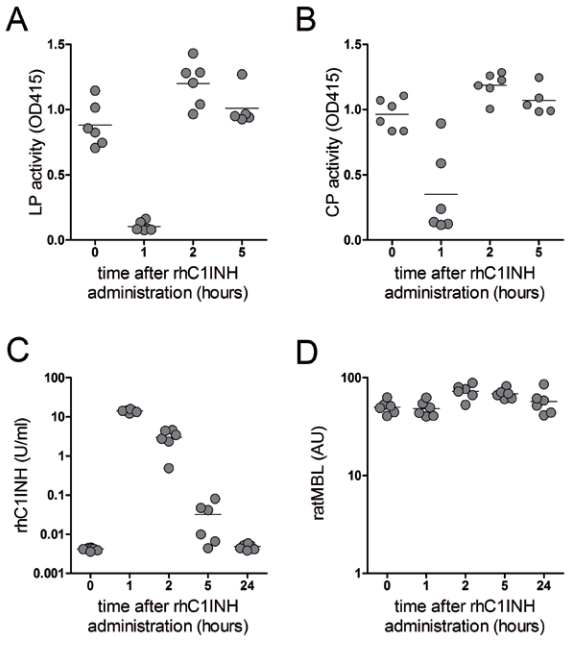

Figure 4. RhC1INH kinetics in rats are unfavorable for effective protection. Before induction of unilateral ischemia and removal of the contralateral kidney, rats received rhC1INH intravenously (625 U/kg). Before and following rhC1INH infusion, blood samples were drawn at consecutive time points to assess lectin (A) and classical (B) pathway activity and circulating levels of rhC1INH (C) and MBL (D).

which are co-purified with MBL, might be involved in MBL-mediated tubular cell death, blocking antibodies to MASP-2 were applied. Exposure of tubular cells to MBL in the presence of MASP-2 antibodies did not prevent MBL-mediated cell death (Fig 5A, B). Since this strategy did not target MASP-1, MASP-free MBL was purified. Using a MBL-MASP complex ELISA, absence of MBL-MASP complexes was confirmed (Fig 5C). Incubation of MASP-free MBL on tubular cells still affected tubular cells (Fig 5B), indicating that MASPs are not involved in MBL-mediated tubular injury.

DISCUSSION

In the present study we demonstrated that rhC1INH is not protective in a rat model of renal IRI. Therapeutic administration of functionally active rhC1INH *in vivo* did not attenuate renal dysfunction following reperfusion, nor did it inhibit influx of inflammatory cells, tubular injury or complement cascade activation.
Although rhC1INH inhibited complement activation *in vitro*, tubular C3 deposition at 24 hours following reperfusion was present in rhC1INH-treated animals at the same extent as control-treated animals. Recently, we and others have shown that activation of complement is a relative late event following reperfusion with the first signs of C3 deposition at 2 and 24 hours upon reperfusion in mice (7) and rats (30), respectively. In our rat model, kinetic analysis revealed that rhC1INH

only effectively inhibited complement activity in the first hour after reperfusion and that both classical as well as lectin pathway activity was fully restored in the next hour. This fast clearance of rhC1INH (Fig 4A) might explain the inability of rhC1INH to reduce complement deposition and complement-mediated injury. Castellano et al (6) showed that a comparable dose of rhC1-inhibitor infused in pigs significantly protected against renal IRI and reduced both lectin and classical pathway activation up to at least 240 minutes after infusion, indicating an important species difference in the clearance of and protection by rhC1INH. Another important difference between these two models is the presence of peritubular C4d staining in pigs following reperfusion (6), which is completely absent in rats (data not shown). This suggests that classical and/or lectin pathway activation by C1q and MBL leading to C4 deposition in the kidney following reperfusion is virtually absent in rodents. These data are in line with IRI studies in mice, in which C4-deficient animals are not protected (10). However, recently

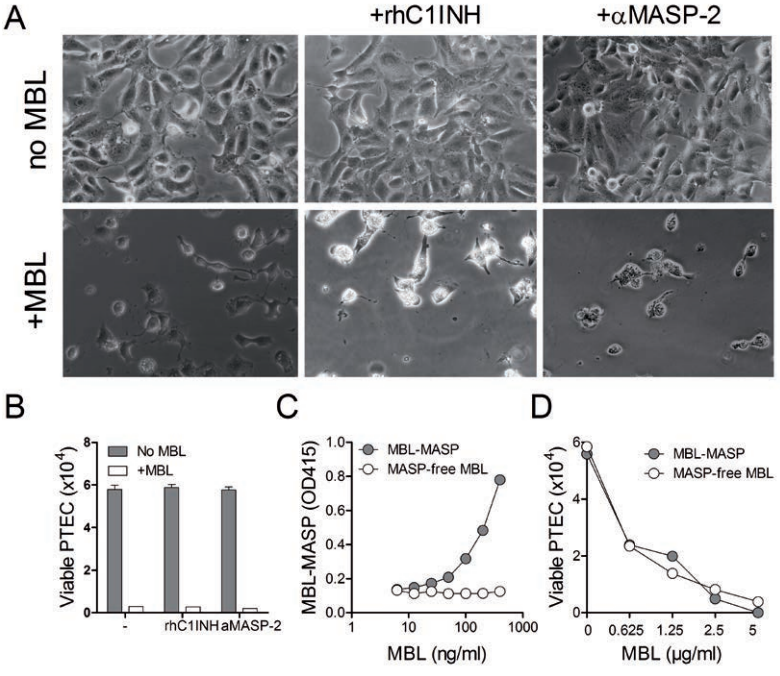

Figure 5. RhC1INH does not inhibit MBL-mediated tubular cell death. Human PTEC (HK-2) were incubated with or without 10 µg/ml human MBL (A, B) in the presence or absence of rhCINH (1 mg/ml) or blocking antibodies to MASP-2 (100 µg/ml) for 24 h. Subsequently, wells were photographed (A; magnification ×200) and quantified (B). To assess the contribution of MASPs in MBL-mediated cell death, MASP-free MBL was generated (C) and incubated (D) in a dose response on human PTEC. Viable cells were assessed by counting Hoechst-positive, but PI-negative cells in the well using fluorescence microscopy.

it was shown in several mouse models of IRI, that MBL in association with MASP-2 (31) can bypass C4 and directly cleave C3 (32). Indeed, mice deficient for MBL were protected against renal IRI (33). Glycosylated tubular meprins, which bind MBL (34;35), were shown to be involved in the activation of complement in the kidney. Inhibition of MBL-MASP complexes by rhC1INH might therefore still be a therapeutic option, however the unfavorable fast clearance of rhC1INH does not warrant any protective effects in rodents.

We recently demonstrated an entirely new concept of lectin pathway participation in renal IRI. In a rat model we showed that MBL exerts direct cytotoxic effects to tubular epithelial cells in the complete absence of complement activation (30). Inhibition of MBL was almost completely protective against renal IRI, whereas downstream inhibition of complement was not. Because rhCINH targets the MBL-associated serine proteases (MASPs) (15), we examined whether MASPs might be involved in this MBL-mediated tubular cell death. However, blockade of MASP-2 or depletion of MASPs from MBL, did not abrogate the cytotoxic effect of MBL (Fig 5), suggesting that interference of rhC1INH with MASPs would not reduce the cytotoxicity of MBL towards tubular cells.

RhC1INH, in contrast to purified human C1INH, is known to be heavily glycosylated (23;24) and is therefore thought to have an additional inhibitory effect towards the lectin pathway of complement by directly binding MBL. Since rhC1INH was cleared quickly from circulation (Fig 4A), we considered whether this might affect circulating levels of MBL. This effect however was negligible since circulating MBL levels following reperfusion remained stable in the first hours following rhC1INH administration. Also *in vitro*, rhC1INH did not inhibit cytotoxic effects exerted by MBL towards human tubular cells, indicating that rhC1INH is not able to reduce the complement-independent effects of MBL.

Although an important function of C1INH is to regulate complement activation, it also inhibits proteases of the fibrinolytic, clotting, and kinin pathways. Deficiency of C1INH is associated with hereditary angioedema (22;36). Involvement of fibrinolytic and clotting pathways in renal IRI has been demonstrated (37-39), which also might be influenced by C1INH. In the past few years, it has become apparent that C1INH has additional anti-inflammatory functions independent of protease inhibition. These include interactions with leukocytes that may result in enhanced phagocytosis (40), with endothelial cells via E- and P-selectins that interfere with leukocyte rolling and in turn results in suppression of transmigration of leukocytes across the endothelium (41) and interactions with extracellular matrix components (42;43). In addition, C1INH has a direct anti-apoptotic activity on vascular endothelial and myocardial cells, associated with blocking of cytochrome c translocation and the inhibition of caspase-3 activation by normalization of the pro-apoptotic Bcl-2/Bax expression ratio

(44;45). Altogether, these effects contribute to the protective effect of C1INH in reperfusion injury. Treatment with C1INH has been successfully performed in animal IRI models in the heart (44;46), intestine (47), skeletal muscle (48), liver (49) and brain (50;51). However in all these models, purified C1INH from human plasma was used. This might explain the discrepancy observed with our renal IRI model, in which the recombinant form of C1INH was used. Pharmacokinetic studies in rats revealed that the recombinant form was cleared from circulation within two hours. Timing of rhC1INH administration before the initiation of ischemia was therefore not optimal, since most rhC1INH has been cleared from the circulation by the time that it was actually needed and complement activation was most evident. Furthermore, the complement-independent MBL-mediated cytotoxicity, which could not be inhibited by rhC1INH *in vitro*, may be more pronounced in the kidney than in other organs systems.
Taken together, we demonstrated that rhC1INH is not protective against renal IRI in rats and does not rescue human tubular cells from MBL-mediated cytotoxicity *in vitro*.

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CHAPTER 8

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Renal ischemia/reperfusion induces a dysbalance of angiopoietins, accompanied by proliferation of pericytes and fibrosis

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ABSTRACT

Introduction: Endothelial cells (ECs) are highly susceptible to hypoxia and easily affected upon ischemia/reperfusion (I/R) during renal transplantation. Pericytes and angiopoeitins play important role in modulating EC function. In the present study, we investigate the effect of renal I/R on dynamics of angiopoietin expression and its association with pericytes and fibrosis development.
Methods: Male Lewis rats were subjected to unilateral renal ischemia for 45 minutes followed by removal of the contralateral kidney. Rats were sacrificed at different time points after reperfusion. Endothelial integrity (RECA-1), pericytes (PDGFRβ), Angiopoietin-2 (Ang-2)/Angiopoietin-1 (Ang-1) expression and interstitial collagen deposition (Sirius Red and α-SMA) were assessed using immunohistochemistry and RT-PCR.
Results: Our study shows an increase in protein expression of Ang-2 starting at 5 hours and remaining elevated up to 72 hours, with consequently higher Ang-2/Ang-1 ratio after renal I/R ($p<0.05$ at 48 hours). This was accompanied by an increase in protein expression of the pericytic marker PDGFRβ and a loss of ECs (both at 72 hours after I/R, $p<0.05$). Nine weeks after I/R, when renal function was restored, we observed normalization of the Ang-2/Ang-1 ratio and PDGFRβ expression and increase in cortical ECs, which was accompanied by fibrosis.
Conclusions: Renal I/R induces a dysbalance of Ang-2/Ang-1 accompanied by proliferation of pericytes, EC loss and development of fibrosis. The Ang-2/Ang-1 balance was reversed to baseline at 9 weeks after renal I/R, which coincided with restoration of cortical ECs and pericytes. Our findings suggest that angiopoietins and pericytes play an important role in renal microvascular remodeling and development of fibrosis.

INTRODUCTION

Renal I/R is an inevitable consequence of renal transplantation causing significant graft injury (5;16;29). Renal I/R impairs the integrity of ECs and leads to loss of peritubular capillaries (6;9;17;19;25;32;36). Loss of integrity and function of the endothelial monolayer lead to renal hypoxia, which is suggested to be a major initiator of profibrotic changes and interstitial scar formation in the kidney (2;24). These microvascular changes and renal scarring eventually lead to a deteriorating of renal function and graft loss (26).
Pericytes play a critical role in the stabilization and proliferation of peritubular capillaries via interaction with ECs (1;20;30). This process is mediated by several angioregulatory factors, including Ang-1, produced by pericytes and Ang-

2 produced by activated ECs (7;30;35). Angiopoietins are a group of vascular regulatory molecules that bind to the receptor tyrosine kinase Tie-2, which is predominantly expressed by vascular ECs. Ang-1 is a strong vascular protective agonist of the Tie-2 receptor responsible for suppressing vascular leakage, maintaining EC 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. By promoting pericyte dropout, Ang-2 will lead to loosening contacts between ECs and perivascular cells, with subsequent vessel destabilization and abnormal microvascular remodeling (7;14;15;35). Recent studies have shown that pericytes detach from the endothelium and migrate to the interstitium to become activated and differentiate into myofibroblasts contributing to renal fibrosis (10;12). Interestingly, treatment with cartilage oligomeric matrix protein (COMP)-Ang-1 in a mice model of renal I/R injury resulted in protection against peritubular capillary damage and decrease in inflammatory cells and renal interstitial fibrosis (19).

However, dynamics and the time course of angiopoietin expression, its relation with EC and pericyte expression and development of fibrosis in the repair phase after renal I/R injury are unknown. Using an established rat model of renal I/R injury, we assessed the impact of I/R on Ang-2/Ang-1 balance and its effect on microvascular remodeling, pericytes and the formation of fibrosis up to 9 weeks after renal I/R injury. We hypothesize that I/R injury leads to activation of ECs with consequent elevation of Ang-2 levels, which may lead to proliferation of pericytes and loss of ECs, but may also induce fibrosis in the long term.

METHODS

Rat model of renal I/R injury

Renal I/R injury was induced as previously described (34). The Animal Care and Use Committee of the Leiden University Medical Center approved all experiments. Eight-week-old male Lewis rats (200–250 g) purchased from Harlan (Horst, The Netherlands) were housed in standard laboratory cages and allowed free access to food and water throughout the experiments. Unilateral ischemia was induced by clamping of the left renal pedicle for 45 min using a bulldog clamp (Fine Science Tools, Heidelberg, Germany). During clamping the contralateral kidney was removed. Sham-treated rats had identical surgical procedures except for clamping of the left kidney. Tail blood samples were taken before and at indicated time points after reperfusion and were kept on ice. Rats were sacrificed at 2, 5, 24, 48 or 72 hours (hr) and 1, 6 and 9 weeks (wk) after reperfusion and kidneys were harvested for histological examination and immunohistochemical staining.

Renal function was assessed by measuring creatinine and urea (BUN) in serum samples using standard auto analyzer methods by our hospital research services.

Immunohistochemistry and immunofluorescent staining

Rat kidney sections (4 μm) of snap-frozen kidneys were air dried and acetone fixed. Slides were incubated overnight with goat polyclonal IgG against Ang-1 (N18) or Ang-2 (F18; both Santa Cruz Biotechnology), mouse monoclonal IgG against endothelial cells (RECA-1; Hycult Biotechnology, Uden Netherlands and CD31; Abcam, Cambridge, England), myofibroblasts (α-SMA, Progen, Heidelberg, Germany), inflammatory cells (OX42+ for monocytes, dendritic cells and granulocytes, and CD45 (BD Pharminghen, Breda Netherlands) for leukocytes) or rabbit polyclonal IgG against pericytes (PDGFRβ; Abcam, Cambridge, England). Antibody binding was detected with horseradish peroxidase (HRP)-labeled rabbit anti-goat IgG (DAKO, Glostrum, Germany), goat anti-mouse IgG (Jackson, Suffolk, England) or goat anti-rabbit IgG (DAKO), respectively. After washing, sections were incubated with tyramide-fluorescein isothiocyanate in tyramide buffer (NENTM Life Science Products, Boston, MA, USA), washed and incubated with HRP-labeled rabbit anti-fluorescein isothiocyanate (DAKO, Glostrum, Germany) and developed with 3,3'-Diaminobenzidine (DAB) (Sigma, St Louis, MO, USA). Sections were counterstained with haematoxylin (Merck, Darmstadt, Germany) and mounted with imsol (Klinipath, Duiven, the Netherlands). Quantification of immunohistochemistry was performed in a blinded manner by assessing consecutive high power fields (magnification, ×100) on each section from the cortex, outer and inner medulla. Using Image J software, the percentage of positivity per specific region of the kidney was determined, with exception of Ang-1 and Ang-2, which was only analyzed for the cortex. Glomeruli were excluded from all analyses of the cortex. Since the Ang-2/Ang-1 ratio, rather than the absolute expression of either angiopoietin is generally used to determine the functional status of the microvasculature (26), this ratio was calculated using the Ang-2 and Ang-1 quantification. Immunofluorescent double stainings were performed for ki-67 (cell proliferation marker) using polyclonal rabbit IgG (Abcam, Cambridge, England) and RECA-1 and Ang-2/RECA-1. Due to technical reasons double stainings with Ang-1/ PDGFRβ and RECA-1/ PDGFRβ could not be performed, therefore the pericyte marker NG2 (rabbit polyclonal IgG; BD Pharmingen, Breda, Netherlands) was used for these double stainings. Antibody binding was visualized using Alexa™ 488-labeled goat anti-rabbit IgG, Alexa™ 568-labeled goat anti-mouse IgG (both Life science) and donkey anti-goat IgG (Jackson, Suffolk, England). Nuclei were stained using Hoechst (Molecular Probes, Leiden, the Netherlands). Micrographs were made using a fluorescence microscope (Leica, DMI6000, Rijswijk, the Netherlands).

Histologic evaluation

Renal fibrosis was evaluated histologically by Sirius Red staining as described previously (27) on 4 µm paraffin slides of renal rat tissue. From each part of the kidney (cortex, outer and inner medulla) five random images were obtained. Image analyses was performed using Image J software. The amount of collagen deposition was measured and expressed as percentage of positivity per region of the kidney. In addition, all tissue specimens were scored for severity of fibrosis on a semi-quantitative scale (0-3) in a blinded manner by an experienced pathologist.

RNA isolation and Real-Time PCR

RT-PCR was performed as described previously (34). Total RNA was extracted from snap frozen cross-section kidney slices using the RNeasy Mini isolation Kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany). cDNA was synthesized from 1 µg total RNA, using an oligo dT primer, RNase-OUT, M-MLV reverse transcriptase, 0.1 M-DTT and buffers in a volume of 20 µL (all purchased from Invitrogen, Breda, The Netherlands). Quantitative real-time PCR was performed in duplicate by using iQ SYBR Green Supermix on iCycler Real-Time Detection System (BioRad). The amplification reaction volume was 20 µL, consisting of 10 µL iQ SYBR Green PCR mastermix, 1 µL primers, 1 µL cDNA, and 8 µL water. Data were analyzed using Gene Expression Analysis for iCycler Real-Time PCR Detection System (Biorad). Expression of each gene was normalized against mRNA expression of the housekeeping gene Rsp-15. RT PCRs were performed in duplicate. The primer sequences are shown in table 1.

Table 1. Primer sequences used for quantitative real-time PCR

Gene	Forward primer 5'->'3	Reverse primer 5'->'3	Supplier
RSP-15	CGTCACCCGTAATCCACC	CAGCTTCGCGTATGCCAC	Biolegio
ANG-1	TCTCTTCCCAGAAACTTCA	TTTGATTTAGTACCTGGGTCTC	Biolegio
ANG-2	TGCATCTGCAAGTGTTCCC	GCCTTGAGCGAGTAACCG	Biolegio
TIE-2	GTCCTATGGTGTATTGCTCTG	TCTCTCATAAGGCTTCTCCC	Biolegio

Statistical analyses

Data are reported as mean ± standard error of the mean (SEM). Statistical comparisons were performed using one-way ANOVA or Mann–Whitney test with GraphPad Prism software (GraphPad Software Inc., San Diego, CA, U.S.A.). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Renal I/R induces transient deterioration of renal function, influx of inflammatory cells and interstitial fibrosis

In the current study we used a rat model of renal I/R resulting in extensive renal dysfunction, as shown by increased serum creatinine and BUN levels, but characterized by normalized renal function after 1 week following I/R ($p<0.05$, compared to 72 hr) (Fig. 1A, B). Renal dysfunction was accompanied by significant

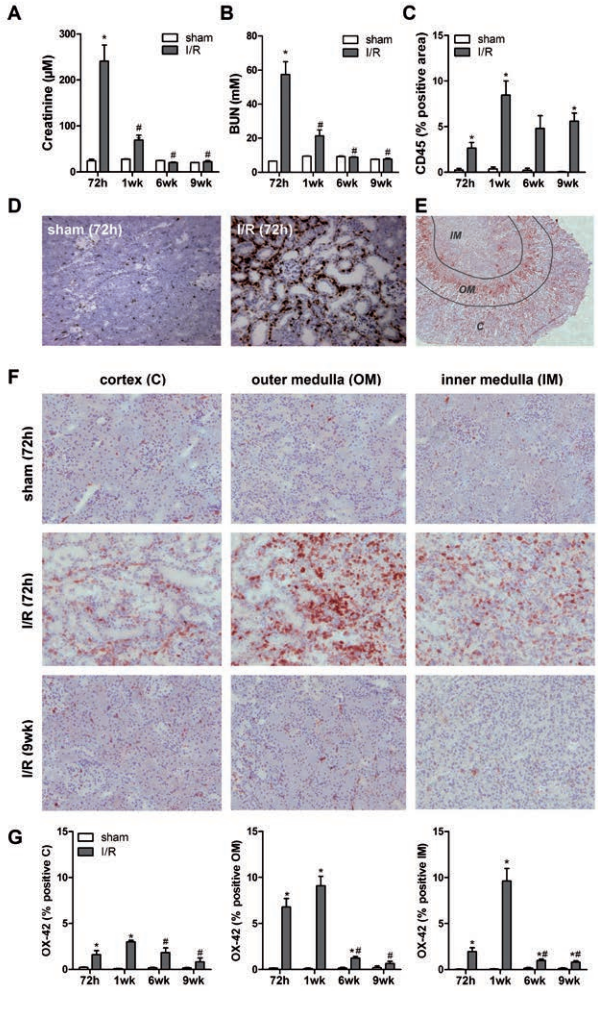


Figure 1. Renal I/R induces deterioration of renal function and influx of inflammatory cells. Serum creatinine levels (A) and BUN (B) were measured at consecutive time points after reperfusion. CD45+ leukocyte infiltrate was quantified using digital image analysis (C). Representative photomicrographs of kidney sections stained with CD45 (D) from a sham-operated rat and a rat subjected to I/R and sacrificed at 72 hr after reperfusion. An overview of the division of the different regions (cortex, outer medulla and inner medulla) in a kidney section stained with OX42 (E). Representative photomicrographs of kidney sections stained with OX42+ from a sham-operated rat and rats subjected to I/R and sacrificed at 72 hr or 9 weeks after reperfusion (F). OX42+ infiltrate of sham-operated rats and rats subjected to I/R was quantified in the different areas using digital image analysis (G) and demonstrated as % of the depicted area. Data are shown as mean ± SEM (n= 5 rats per group). *P<0.05 compared to corresponding sham controls. #P<0.05 compared to 72 hr or 1 wk rats. Original magnification of D, E and F, x200. C=cortex; OM=outer medulla; IM=inner medulla.

infiltration of OX42+ inflammatory cells in the cortex, outer and inner medulla at 72 hr ($p<0.05$), which peaked at 1 week compared to sham-operated rats (Fig. 1E, F, G). A decrease in OX42+ inflammatory cells was observed at 6 and 9 weeks in the different parts of the kidney compared to 1 week following I/R ($p<0.05$). In the outer and inner medulla, the influx of OX42+ cells remained significantly increased up to 6 and 9 weeks ($p<0.05$), respectively, after renal I/R injury

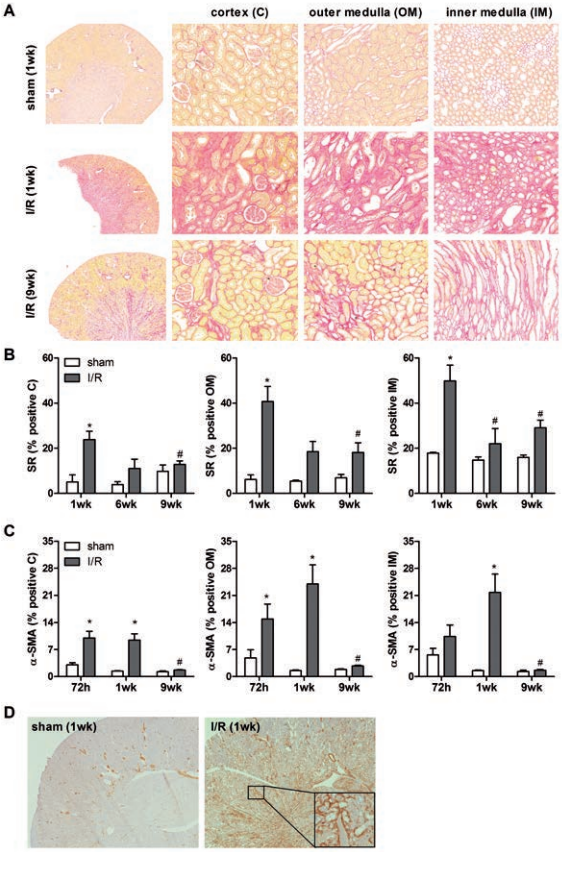


Figure 2. Renal I/R induces interstitial fibrosis. Representative photomicrographs of Sirius Red stained kidney sections (A) of the cortex, outer medulla and inner medulla obtained from a sham-operated rat (sacrificed at 1 week), and rats subjected to I/R and sacrificed at 1 or 9 weeks after reperfusion as indicated. Quantitative analysis of Sirius Red staining (B) and α-SMA staining (C) at different time points after reperfusion in sham-operated rats and rats subjected to I/R in the different regions as indicated. Quantification was performed by digital image analysis and is demonstrated as % of the depicted area. Data are shown as mean ± SEM (n= 5 rats per group). *P<0.05 compared to corresponding sham controls. #P<0.05 compared to 72 hr or 1 wk rats. Original magnification of A, x200. C=cortex; OM=outer medulla; IM=inner medulla.

compared with sham-operated rats (Fig. 1G). Consistently, CD45 expression in the cortex showed a similar pattern as OX42+ staining (Fig. 1C, D).

One week after reperfusion, significant diffuse interstitial collagen deposition was observed in the cortex, outer and inner medulla compared to sham-operated rats ($p<0.05$) (Fig. 2A, B). After 9 weeks collagen deposition was significantly decreased in different regions of the kidney compared to 1 week following renal I/R injury ($p<0.05$), although kidneys were still characterized by focal areas of intense Sirius Red staining. The semi-quantitative analyses showed that fibrosis scores at 9 weeks were not statistically different from 1 week after I/R (data not

shown), which is probably due to the focal areas of fibrosis. Consistently, the α-SMA immunohistochemical staining revealed a significant increase of fibrosis in cortex and outer medulla at 72 hr ($p<0.05$) and at all parts of the kidney at 1 week ($p<0.05$) following I/R injury compared with sham-operated rats (Fig. 2C, D). At 9 weeks, α-SMA staining was significantly decreased in the cortex, outer and inner medulla compared with 1 week after I/R injury (Fig. 2C).

Restoration of peritubular capillaries in the cortex after renal I/R

Since endothelial damage is an important hallmark of I/R injury, we assessed peritubular capillaries over time by staining for RECA-1. A significant reduction in RECA-1 expression was observed at 72 hr post I/R ($p<0.05$) in the cortex and

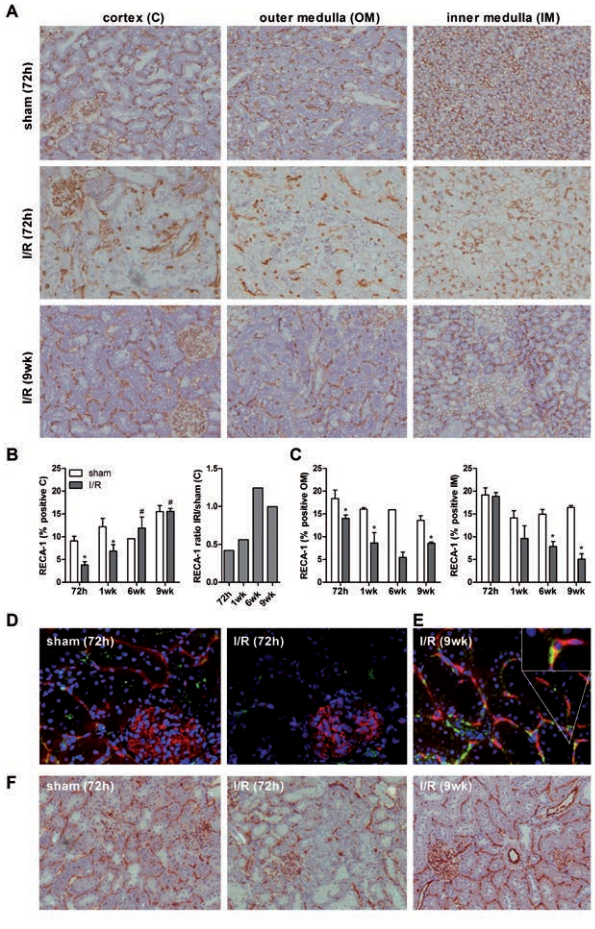


Figure 3. Restoration of I/R-induced peritubular capillary loss in the cortex late after reperfusion.
Representative images of kidney sections of the cortex, outer medulla and inner medulla stained for RECA-1 (A) obtained from a sham-operated rat and rats subjected to I/R and sacrificed at 72 hr and 9 weeks after reperfusion. RECA-1 was quantified using digital image analysis as indicated (B). Immunofluorescent double staining for RECA-1 (red) and Ki-67 (green) of representative kidney sections of a sham-operated rat and rats sacrificed at 72 hr (C) or 9 weeks (D) after reperfusion. Insert is showing double positivity of RECA-1 and Ki-67 staining in yellow in kidney sections at 9 weeks after reperfusion. Representative images of kidney sections of the cortex stained for CD31 (E) obtained from a sham-operated rat and rats subjected to I/R and sacrificed at 72 hr or 9 weeks after reperfusion. Data are shown as mean ± SEM (n= 5 rats in grouper group). *P<0.05 compared to corresponding sham controls. #P<0.05 compared to 72hr. Original magnification of A, C and E, x200; insert at 9 weeks, x400.

outer medulla and at 1 week in the inner medulla ($p<0.05$) compared with sham-operated rats (Fig. 3A, B). Interestingly, we found a significant increase of RECA-1 staining at 6 and 9 weeks in the cortex ($p<0.05$) following I/R compared to rats that were sacrificed at 72 hr (Fig. 3B). RECA-1 staining colocalized with the proliferation marker Ki-67, suggesting EC proliferation in peritubular capillaries within the renal cortex at 9 weeks after I/R (Fig. 3C). No proliferation of ECs was found in the glomeruli following renal I/R. In the outer and inner medulla a different pattern was found. Here, a significant reduction in RECA-1 expression was observed at 72 hr post I/R in the outer medulla ($p<0.05$) and at 1 week in the inner medulla ($p<0.05$) compared with sham-operated rats (Fig. 3B). In contrast to the cortex, RECA-1 staining in these areas showed a further decrease up to 9 weeks after ischemic injury ($p<0.05$) (Fig. 3B). CD31 staining demonstrated a pattern comparable to the RECA-staining (Fig. 3E).

Renal I/R induces a dysbalance in Ang-2/Ang-1 ratio at early time points and return to baseline after 6 weeks

In control kidneys of sham-operated rats, Ang-1 staining was observed in the glomeruli and in a capillary like pattern between the tubuli (Fig. 4A). Double staining of Ang-1 and the pericyte marker NG2 revealed colocalization, suggesting that Ang-1 is expressed by pericytes (Fig. 4C). Starting at 24 hr after renal I/R, a significant decrease in Ang-1 expression was observed (data not shown), reaching a maximal decrease at 72 hr ($p< 0.05$) (Fig. 4A, F). Ang-1 expression increased significantly at 9 weeks after renal I/R compared to 72 hr ($p<0.05$) (Fig. 4A, F). Additionally, RT-PCR analyses revealed a decrease in Ang-1 mRNA levels which reached significance at 1 week compared the sham-operated rats ($p<0.05$; Fig. 4I). In control rats, low levels of Ang-2 protein were observed in glomeruli, interstitial vessels and on brushborders of tubuli (Fig. 4B, G). Due to the apical and brush border expression on tubuli of Ang-2 in the medulla, it was technically not possible to distinguish between interstitial and tubular presence of Ang-2 (Fig. 4E). Therefore quantification of angiopoietins was only performed in the cortex. Additional double staining of Ang-2 and RECA-1 confirmed the expression of Ang-2 by ECs (Fig. 4D). Compared to sham-operated rats, Ang-2 expression increased at 5 hr (data not shown) and remained elevated up to 72 hr ($p< 0.05$) after I/R (Fig. 4G). Consequently higher Ang-2/Ang-1 ratios were observed after 48 hr and 72 hr ($p<0.05$) (Fig 4H). After 1 week, Ang-2 levels and Ang-2/Ang-1 ratio started to decrease, reaching significance at 6 and 9 weeks compared to 72 hr after renal I/R (Fig. 4B, G and H). Ang-2 mRNA levels did not confirm the observed changes at protein levels (Fig. 4J). Tie-2 mRNA expression showed a significant decrease at 1 week compared with sham-operated group and reversal to baseline levels at 9 weeks after I/R (Fig. 4K).

Renal I/R leads to proliferation of pericytes

Immunohistochemical staining revealed the presence of PDGFRβ positive cells in the mesangium, Bowman's capsule, large vessels and peritubular capillaries (Fig. 5A). A significant increase of pericytes was observed at 48 hr upon I/R in all parts of the kidney compared with sham-operated rats, which persisted up

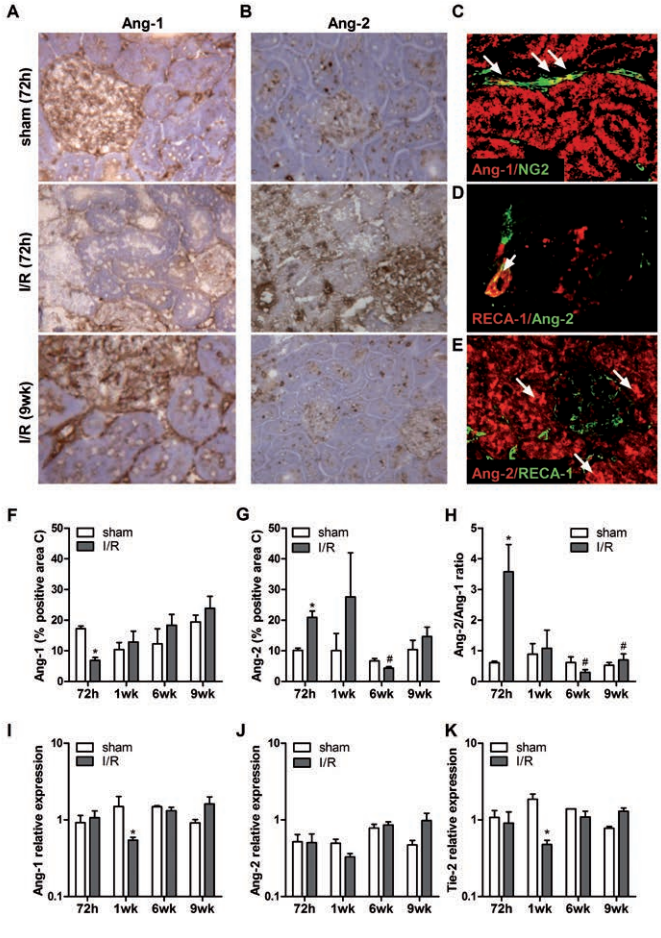
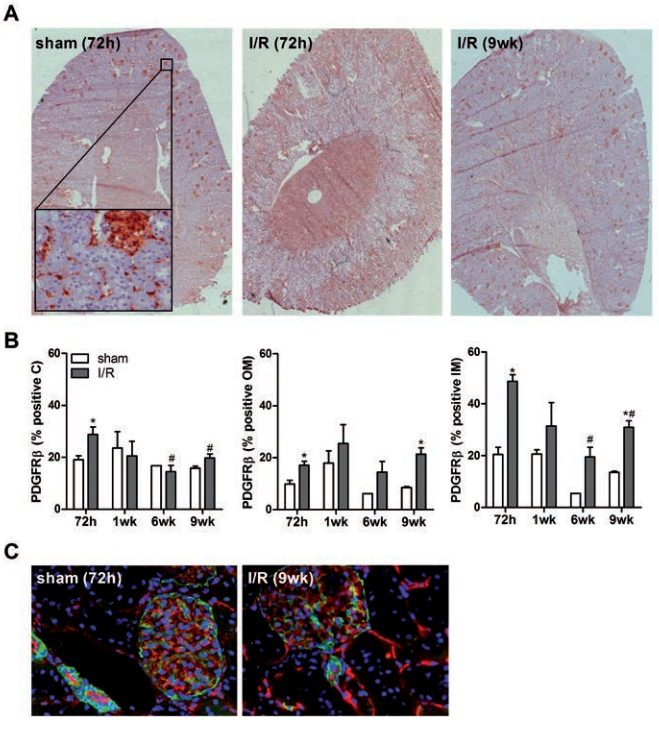


Figure 4. Renal I/R induces a dysbalance in the Ang-2/Ang-1 ratio early after reperfusion. Representative cortical photomicrographs of kidney sections stained with Ang-1 (A) or Ang-2 (B) in a sham-operated rat and rats subjected to I/R and sacrificed at 72 hr or 9 weeks after reperfusion. Immunofluorescent double staining for Ang-1/NG2 (C), RECA-1/Ang-2 (D) and Ang-2/RECA-1 (E) of representative kidney sections in a sham-operated rat. Arrows indicate double staining in yellow (C,D) and apical expression of Ang-2 (E). Quantitative analysis of cortical protein expression of Ang-1 (F), Ang-2 (G), and Ang-2/Ang-1 ratio (H) at consecutive time points after reperfusion. Quantitative analysis of RNA expression of Ang-1 (I), Ang-2 (J) and Tie-2 (K) at consecutive time points after reperfusion. Data are shown as mean ± SEM (n= 5 rats per group). *P<0.05 compared to corresponding sham controls. #P<0.05 compared to 72 hr. Magnification of A-E, x400.

to 72 hr in the cortex and up to 9 weeks in both the outer and inner medulla (p< 0.05) (Fig. 5A, B). PDGFRβ expression showed normalization at the protein level in all regions of the kidney (p<0.05) at 9 weeks after renal I/R injury (Fig. 5A, B). To investigate the anatomical relation of ECs and pericytes double staining

with RECA-1 and the pericyte marker NG2 were performed, which revealed expression of both markers in the glomeruli and peritubular space (Fig. 5C).

Figure 5. Renal I/R induces proliferation of pericytes. Representative photo-micrographs of whole kidney sections stained for PDGFRβ (A) in a sham-operated rat and rats subjected to I/R and sacrificed at 72 hr or 9 weeks after reperfusion. Insert is showing peritubular and glomerular expression of PDGFRβ in sham-operated rats. Quantitative analysis of PDGFRβ protein expression (B) at consecutive time points after reperfusion. Fluorescent double staining for RECA-1 (red) and NG2 (green) of representative kidney sections (C) in a sham-operated rat and rat subjected to I/R and sacrificed at 9 weeks after reperfusion. Data are shown as mean ± SEM (n= 5 rats per group). *P<0.05 compared to corresponding sham controls. #P<0.05 compared to 72hr. Original magnification of A, x100; insert at 72hr and C, x200.



DISCUSSION

We performed a detailed kinetic analysis of angiopoietin and pericyte expression after renal I/R injury. We demonstrate that renal I/R induces a dysbalance in Ang-2/Ang-1 which is accompanied by a loss of ECs, proliferation of pericytes and development of fibrosis. At 9 weeks post I/R, we show reversal to baseline in Ang-2/Ang-1 balance, an increase in ECs and normalization of PDGFRβ expression in the cortex. Whereas renal function is fully restored at this time point, the renal tissue does still show signs of fibrosis.

It is postulated that loss of ECs is a central common pathway involved in organ failure that precedes and drives the profibrotic changes of the kidney parenchyma (2;3;24). Different reports using animal models have been

published which demonstrate chronic deleterious effects of ischemic injury on long-term renal function and microvascular structure (2;6;16;17;29;36). Basile et al have shown that renal I/R in a rat model results in permanent damage to peritubular capillaries with development of tubulointerstitial fibrosis and decline in long-term renal function (2). In the present study, we also observed loss of peritubular capillaries and development of tubulointerstitial fibrosis following renal I/R, which were more severe and extensive in the outer and inner medulla than the cortex. The observed fibrosis at early time points after renal I/R injury in our study coincided with the influx of inflammatory cells. At later time points, we observed a different pattern of fibrosis, which was more dense and unrelated to the areas of inflammation. This 'inflammatory fibrosis' observed at early times has been reported to be important for restoration of the original tissue morphology and function (13). It is, however, also suggested that if repair is not efficient at early times, fibrosis at the repair phase cannot be prevented (9). In our study, at 9 weeks after reperfusion, restoration and proliferation of ECs was found in the cortex, but not in the outer and inner medulla. This could be caused by the anatomical relationship of capillaries and tubules in the outer and inner medulla, with consequently a greater impact of hypoxia- and leucocyte-induced EC damage than in the cortical peritubular capillaries (5).

In contrast to the study of Basile et al, we found restoration and proliferation of ECs in the cortex in the repair phase (9 weeks after I/R). Possible explanations for this discrepancy between our study and the studies of Basile could be differences in clamping time (45 versus 60 minutes, respectively) and the removal of the healthy contralateral kidney in our study. In the study of Basile, additional experiments in which rats were subjected to 30 and 45 minutes of bilateral ischemia were performed, to assess whether the duration of ischemic injury affects damage to the renal microvasculature and function. Both renal function and capillary density were more disturbed in the 45 minutes group compared to the 30 minutes rats, which might implicate a "critical ischemia time" for endothelial repair (2).

The molecular mechanisms that lead to microvascular injury in organ failure are largely unknown. It has been suggested that several angioregulatory growth factors, including the angiopoeitins, play a central role in the loss of vascular integrity (18;36). In this regard, a study has shown that treatment with COMP-Ang-1 (soluble, stable and potent form of Ang-1) in a mice model of renal I/R resulted in protection against peritubular capillary damage, decrease in interstitial inflammatory cells and renal interstitial fibrosis (5). Other investigators have demonstrated stabilization of peritubular capillaries along with increased fibrosis and inflammation after adenoviral Ang-1 treatment in a mouse model of folic acid-induced nephrotoxicity (37). These studies suggest that differences

in efficacy of Ang-1 in the kidney may be due to variation in potency of Ang-1 and COMP-Ang-1 or difference in kidney disease models (5;12;37). Ang-2 acts as an antagonist of Ang-1 and increases vessel leakage, inflammation and destabilization by promoting pericyte loss, therefore loosening contacts between ECs and pericytes (18;36).

An interesting observation in this study is the relation in time between Ang-2/Ang-1 balance and microvascular integrity and pericytes in the cortex after I/R. Activation of ECs, reflected by increase in Ang-2 expression and consequently higher Ang-2/Ang-1 ratio, was accompanied by proliferation of pericytes, EC loss and development of fibrosis. This relation between EC loss and dysbalance in angiopoietins has also been demonstrated in a mouse model of anti-glomerular basement membrane glomerulonephritis, where glomerular capillary loss was associated with reduced Ang-1 and increased Ang-2 expression (38). In addition, in an animal model of diabetic retinopathy, Ang-1 was shown to have a profound effect in repairing integrity of the retinal EC permeability barrier (39). Moreover, injection of Ang-2 into the eyes of normal rats has been shown to induce a dose-dependent pericyte loss (28). These findings suggest an important role for the angiopoeitins in generating a proangiogenic environment that is necessary for capillary repair.

Several studies have pointed at the critical importance of the interaction between pericytes and ECs in maintenance of the capillary network (1;30). Surprisingly, hardly any data are available on the relation between pericytes and loss of ECs in renal I/R. Only one study has shown an association between damage to peritubular capillaries and decreased number of pericytes in cadaveric renal allografts after I/R (21). Peritubular capillary integrity was better preserved and pericytes were more pronounced in patients who had a better recovery of their graft function compared to patients with sustained postischemic acute kidney injury (21). However, this study investigated the expression of pericytes at only one time-point early after renal I/R and did not investigate the relation to angiopoietins and development of fibrosis in a time course.

To investigate the role of pericytes in renal I/R injury, we used PDGFRβ as pericyte marker. PDGFRβ is a single-spanning transmembrane glycoprotein that binds to its dimeric ligand PDGF and a crucial receptor for recruitment and survival of pericytes by paracrine secretion of PDGFB by ECs (4;23;33). PDGFRβ has been shown in studies of obstructive and post-ischemic kidney injury to be expressed by pericytes and fibroblasts (8;22;23). Compared with other pericyte markers, PDGFRβ continued to be expressed after proliferation of pericytes and after transformation into myofibroblasts upon injury. Recently, Duffield et al provided evidence for the contribution of pericytes to the development of renal fibrosis (11;18;23;30). Using a transgenic mouse model of unilateral ureter obstruction

(UUO), expressing green fluorescent protein in cells producing the collagen type I, they demonstrated that pericytes are the main source for interstitial myofibroblasts during renal fibrosis (23). The same investigators showed migration of perivascular stromal cells from capillaries into the renal interstitium, within 9 hr after induction of ureter obstruction. After loosening contact from the capillaries, pericytes became activated and proliferated into collagen producing myofibroblasts contributing to fibrosis (23). PDGFRβ signaling has been reported to play an important role in the development of fibrosis. Blockade of PDGFRβ attenuated recruitment of inflammatory cells, loss of ECs and fibrosis in mice subjected to renal I/R injury and UUO (22). Also in our study we demonstrated proliferation of pericytes and loss of ECs which was accompanied by development of fibrosis. The pericytes may have responded to injury by detaching from the capillaries and becoming pathologic matrix depositing cells, that contribute to the population of α-SMA–positive cells in the fibrotic interstitial space observed in this study (8;31). The process of pericyte detachment has been suggested to be reversible, which could explain the observed decrease in PDGFRβ expression and the restoration of ECs in the cortex (22). However, in both the inner and outer medulla an increase of PDGFRβ cells was observed up to 9 weeks after renal I/R. Interestingly, these areas had no increase in EC staining and demonstrated a more profound fibrosis reaction compared to the less damaged cortex area.

An important discussion point remains whether the decrease in RECA-1 in the cortex observed in our study is explained by EC loss or by interstitial edema and compression of peritubular capillaries. However, previous studies utilizing microfilm analyses and EC staining with CD31 confirmed the loss of ECs after I/R (2;3;17;19;32). Additional CD31 staining in this study revealed a similar pattern as observed with RECA-1. Although our study clearly suggests that angiopoietins are essential in renal microvascular remodeling in the cortex, we were not able to analyze the outer and inner medulla of Ang-2 staining. Ang-2 showed brush border and apical expression on tubules in the medulla, making it difficult to distinguish between interstitial and tubular presence of Ang-2. Therefore, we focused only on the cortex for angiopoietins staining. Furthermore, we observed a discrepancy in Ang-2 protein and mRNA expression, which could be explained by the use of whole kidney RNA extractions instead of a specific region as for immunohistochemistry or contribution of earlier produced Ang-2 by infiltrating cells, while lacking the detectable transcript (28). A therapeutic intervention would be required to prove a causal relationship between the functions of angiopoietins and pericytes and its role in EC stabilization and repair.

In conclusion, our study demonstrates that renal I/R induces a dysbalance in angiopoietins, accompanied by proliferation of pericytes and development of fibrosis. These findings support the hypothesis that angiopoietins and pericytes

play an important role in renal microvascular remodeling. Since angiopoietins and pericytes are considered as important hallmarks of microvascular integrity, strategies to counteract microvascular destabilization after I/R may well improve long term graft function.

GRANTS

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CHAPTER 9

General discussion and summary

9.1 INTRODUCTION

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. 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). Renal I/R is the major cause of acute kidney injury (AKI). AKI is a clinical syndrome characterized by a rapid decrease in renal function and plasma accumulation of creatanine, urea, metabolic acids, potassium and phosphate. 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 ischemic 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.

In the renal transplant setting, ischemia during the transplant procedure, under toxic therapautical conditions or immunological injury, compromise tissue viability and promote 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 (7-11). Ischemic AKI following renal I/R is characterized by injury to the proximal tubular epithelial cells (PTEC) mostly in the cortico-medullary junction. This process is called acute tubular necrosis (ATN). The injured tubular cells are important for tubular reabsorption, the process by which filtered metabolites e.g. salts, proteins and glucose, are reabsorbed from the primary filtrate via the brush border of these cells and transported back into the blood. During an ischemic event, there is shedding of the proximal tubular brush border within several minutes (13) and loss of polarity with mislocalization of adhesion molecules, complement regulators and other membrane proteins (12;13). In advanced ischemic injury, viable and necrotic tubular epithelial cells detach

from the basement membrane. These sloughed tubular cells, brush-border vesicle remnants and cellular debris form characteristic tubular casts, which might activate complement. The complement system is therefore an important contributor to inflammation after IRI. As a consequence of renal ischemia, there is an altered localization and expression of complement regulators on tubular cells (14), which makes these cells prone for complement activation. In addition, apoptotic as well as necrotic cells and tubular casts generated during I/R are potent activators of the complement system (15-18).

9.1.1 Scope of this thesis

Several lines of evidence support an important role for complement in renal IRI. Most of this evidence however, is based on studies performed in mice, which demonstrate a predominant role for the alternative pathway of complement (19-21). In the kidney, the cortico-medullary junction harboring the proximal tubular cells (PTEC) is the region, where most complement activation and deposition of C3 and C5b-9 occurs. The loss of complement regulators at the basolateral surface of tubular cells allows complement activation following I/R on these cells. (14). Although complement activation is a clear hallmark of renal IRI in mice, the involvement of complement activation in human IRI remains to be explored. In addition, the contribution of the different complement pathways in human IRI has not been delineated.

In the past, it has been shown that high levels of MBL are associated with worse allograft survival following clinical kidney transplantation, suggesting a harmful role for MBL and the lectin pathway of complement. Whether MBL might play a harmful role in the early phase of kidney transplantation during renal I/R, remains to be explored. Together, these subjects form the scope of this thesis. In the first part of the thesis (chapter 2-4) we focused on the role of complement and the activation pathways involved in human IRI. In addition, we made an approach to measure human complement activation locally in the renal tissue, in circulation and in the urine early following clinical kidney transplantation. In the second part (chapter 5-7) we focused on the role of MBL and a therapeutic approach to target MBL in the setting of renal IRI. Finally, we describe (chapter 8) how I/R and the mediators involved affect the endothelial compartment, which enables MBL to reach the tubular compartment and injure tubular cells.

9.2 COMPLEMENT IN HUMAN IRI

Most of the IRI studies performed in mice show a predominant role for the alternative pathway of complement (19-21). However, direct and in-depth

studies in humans confirming these findings are lacking and so far in human renal IRI, the activation pathways of complement by ischemic PTEC are incompletely elucidated. To successfully develop therapeutic interventions targeted towards complement activation in human, it is essential to establish the validity of murine data relative to what takes place in the human situation.

9.2.1 Human complement activation *in vitro*

Thus far, it was unknown whether experimental data obtained in mice might be extrapolated to humans. This prompted us to study whether complement is activated and which pathways of complement activation are initiated by human PTEC following I/R and to compare these findings to mouse PTEC (chapter 2). For this purpose, we developed an *in vitro* model to induce hypoxia and subsequent reoxygenation to simulate I/R on human and mouse PTEC. Following reoxgygenation, we studied the role of the different pathways of complement activation. Exposure of human or mouse PTEC to hypoxia followed by reoxygenation in serum as a source of complement, resulted in extensive complement activation. Mouse PTEC exclusively activated complement via the alternative pathway, which was completely in line with *in vivo* IRI studies previously performed in mice (19;21). In contrast, complement activation by human PTEC was exclusively dependent on the classical pathway, supported by studies in C1q-depleted serum and use of blocking antibodies to human C1q. The activation of the classic pathway was mediated by IgM through interaction with modified phosphomonoesters exposed on human hypoxic PTEC. It is known from several studies that late apoptotic and necrotic cells bind natural IgM antibodies, which will lead to activation of the CP (22;23). We could demonstrate a similar binding of IgM to hypoxic human PTEC. Using IgM-deficient serum we showed that complement activation by hypoxic cells also occurs via binding of IgM, probably to phosphorylcholine residues exposed on hypoxic cells, as shown by inhibition of complement activation using phosphorylcholine. In line with these findings, it was recently shown that cultured pancreatic islets bind natural IgM and also activate the CP, probably caused by hypoxia or injury during the culture or harvesting procedure.

Data from studies in mice but also human indicate that complement activation following I/R in skeletal muscle (24), heart (25), intestine (26) and limb (27) *in vivo* also occurs through binding of natural IgM. However, in renal IRI in mice it has been shown that immunoglobulins do not play such a role (28). These findings are in line with reports showing that C4-deficient mice were not protected against renal IRI (21), so it appears that in mice, renal IRI can proceed independently of C4 and immunoglobulins. The reason for this difference compared to human PTEC is unknown so far.

In a pig model however, it was recently shown that the classical and lectin pathways of complement were involved in renal IRI (29). Importantly, this study demonstrated reduced ischemic injury when pigs were treated with C1 inhibitor, a specific inhibitor of both the classical and lectin pathway (30;31). These porcine data are in line with our findings on human tubular cells, which activated the classical pathway of complement. Interestingly, this study (29) also demonstrated that the classical and lectin pathway were activated in human renal transplant recipients suffering from delayed graft function (DGF). The co-localization of activated C4 with both C1q and MBL in graft biopsies obtained from these patients indicated that both these pathways were activated on peritubular capillaries, within the interstitium, and on the glomerular endothelium. However, in this study no C4 staining on tubular epithelial cells was observed, which could be explained by the fact that only cortical biopsies were taken, whereas tubular injury following reperfusion is mostly observed in the cortico-medullary junction of the kidney. In addition, biopsies were taken at 7-15 days after transplantation, in which tubular injury most probably has been cleared and repaired. Due to safety concerns however, biopsying early after reperfusion and specifically in the corticomedullary region is difficult. Previous studies on IRI in humans mostly relied on peripheral blood measurements, which lack sensitivity and do not discriminate between the release of factors from the allograft and systemically released factors. For these reasons, in-depth studies on the role and kinetics of reperfusion-induced complement activation in human have been hampered sofar.

9.2.2 Human complement activation in circulation

Recently, a renewing approach was established (32), which enabled us to measure circulating complement activation products directly released from the reperfused kidney and thereby avoiding the limitations of systemic measurements in human (chapter 3). Via a small catheter placed in the renal vein, blood aliquots were sampled at consecutive time points after reperfusion. In addition, paired arterial blood samples were collected. We concentrated on measurement of sC5b-9 because it is the common endpoint of all three pathways. Furthermore, it is suggested that in mice specifically C5b-9 is essential in the induction of tubular damage in renal IRI (21). For these reasons, arteriovenous concentration differences of terminal sC5b-9 were assessed directly over the reperfused organ to reveal the local activation of complement in human IRI.

Release of sC5b-9 in living donor (LD), brain-dead donor (BDD), and cardiac dead donor (CDD) kidney transplantation were compared. A significantly higher rate of delayed graft function (DGF) was observed in BDD and CDD kidneys compared with LD kidneys. We demonstrated that mostly from CDD grafts, which are most

severely affected by I/R, sC5b-9 was released directly after reperfusion, indicative of intravascular terminal complement activation. sC5b-9 was released only very transiently, directly after reperfusion. This may represent a washout effect. The complement system may be triggered on encounter with intravascular cellular debris accumulated during the cold ischemic period or by encounter with hypoxic or injured endothelium (33;34). Intravascular thrombosis and clot formation during warm ischemia, especially in CDD kidneys might occur. The clotting and complement system are tightly intertwined (35-39). Activated thrombin as well as formed fibrin clots have been shown to activate complement leading to C5b-9 formation. Indeed, potent thrombin activation was demonstrated directly following reperfusion by measuring prothrombin fragment (F)1 and 2 levels, indicative of conversion of prothrombin into thrombin by factor Xa (32). This thrombin activation might also be the cause of the transiently released sC5b-9 observed directly after reperfusion.

A limitation of our study was the fact that the sampling time was restricted to maximally 30 minutes following reperfusion. Except for the very transient release of C5b-9 instantly after reperfusion, there was no release of C5b-9 during the entire sampling period of 30 minutes. The absence of complement activation is in line with mouse IRI experiments, in which the first membrane attack complex elements C6 and C9 are observed only after 12 and 24 hours of reperfusion, respectively (40). Also in our rat IRI model, we observed the first signs of C5b-9 deposition after 24 hours of reperfusion (41), indicating that terminal complement activation is a relatively late event following reperfusion in human as well as in rodents.

Finally, the possibility remains that the complement cascade was transiently activated in living donor kidneys as well, without leading to terminal complement activation. Therefore, release of C5a from the reperfused kidney was assessed, because C5a is more upstream in the complement cascade than the terminal complex C5b-9 is. In agreement with C5b-9 measurements, there was no C5a release from living donor kidneys. This excludes early complement activation after reperfusion and also excludes early involvement of C5a, which has also been ascribed as harmful role in IRI (42).

9.2.3 Human complement activation in renal tissue

Besides the intravascular sC5b-9 formation, C5b-9 could be also formed locally in the kidney without any release into the circulation. To assess local complement activation on tubular cells, tissue content and distribution of C5b-9 in needle biopsies collected before and after reperfusion was assessed. Surprisingly, the tubular surface, where C5b-9 is expected to deposit (21), showed no deposition of C5b-9 in LD, BDD or CDD kidneys after reperfusion. In contrast, renal tissue

of a patient with acute graft rejection showed extensive C5b-9 deposition in the tubular compartment. These findings were supported by a study of Haas et al. (43), where in 1 hour post-transplantation biopsies, no complement deposition as a consequence of reperfusion was detected either. However in this study, only cortical biopsies were collected, whereas complement activation on tubular cells deeper in the cortical-medullary region might have been missed. Therefore, in our study needle biopsies were taken, which make it possible to sample deeper into the kidney. Because these needle biopsies are relative thin, we cannot fully exclude a sampling error in which the most affected area still have been missed. Nevertheless, in more than thirty needle biopsies collected no C5b-9 deposition could be detected, making these findings quite reliable.

9.2.4 Human complement activation in urine

The third approach we applied to study the role of complement in human renal IRI, was to assess complement activation products in the urine in the early post-transplant period. Increased glomerular permeability to large plasma proteins (proteinuria) is common in the early period following renal transplantation, with a prevalence of 15% to 30% at 1 year post-transplantation (44). Complement activation at the tubular epithelial surface of the kidney, which lacks several important complement regulators (CD46, CD55) (45), is considered to be a mediator of tubular injury in the proteinuric condition. In proteinuria, complement proteins, which normally are retained in circulation, are able to pass the glomerular filter barrier, end up in the tubular lumen and are activated by the unprotected epithelial surface of the tubuli (46-48). To this end, the detection of soluble (s)C5b-9 in urine is considered as a clinical indicator of tubular complement activation (49-51), however in the setting of renal IRI in human this has not been assessed before. Therefore, we collected urinary samples at consecutive days following kidney transplantation in a cohort of deceased cardiac dead donors and assessed protein and sC5b-9 content (chapter 4). In line with proteinuria studies, we were able to confirm the relation of urinary sC5b-9 and proteinuria. In the early post-transplant period we found high urinary levels of sC5b-9, significantly correlating with the degree of proteinuria, suggesting activation of filtered complement components at the tubular epithelial surface of the kidney. However, when mimicking proteinuria *in vitro* by exposing serum (or blood) to urine (both negative for sC5b-9), we found extensive generation of sC5b-9 in urine. This process was inhibited by EDTA, confirming activation of the complement system. Removal of remaining cells and cellular debris by filtering the urine reduced the sC5b-9 generation in the urine by half, suggesting that sC5b-9 generation in proteinuric urine is partially caused by cellular debris and dead epithelial cells. This implies that following kidney

transplantation, proteinuria but also procedure-related (microscopic) hematuria may cause urinary complement activation resulting in high urinary sC5b-9 levels. Centrifugation or filtration following collection of patient urines would not prevent this, since urinary complement activation is rapid and most probably already occurs in the urinary tract. So, although sC5b-9 is an attractive urinary biomarker, one should be aware of the risk of extra-renal complement activation independent of a renal contribution. Although urinary sC5b-9 was detected in the majority of renal allograft recipients and significantly correlates with the degree of proteinuria, the implications and clinical relevance of measured sC5b-9 are rather unpredictable and therefore could not be used to draw any further conclusions from the urinary sC5b-9 measurements.

9.3 MBL IN RENAL IRI

Besides a predominant role for the alternative pathway of complement, also involvement of the lectin pathway, in particular by MBL, in the course of renal IRI in mice has been demonstrated. Mice subjected to renal I/R display evident renal MBL deposition on tubular cells which co-localizes with depositions of C3, C6 and C9. The degree of early deposition of MBL is associated with later complement activation, neutrophil influx and organ failure (40). Therefore, a role for MBL in complement activation following I/R was suggested.

Also in human biopsies, deposition of MBL have been observed after kidney transplantation (29) and low pretransplantation levels of MBL are associated with better graft survival after deceased-donor kidney transplantation at the long term (52). Of interest, MBL-deficient mice are protected against renal IRI and MBL-deficient mice reconstituted with recombinant human MBL show a dose-dependent increase of renal injury comparable to the severity in wild-type mice (53).

Remarkably in mice, the downstream classical and lectin pathway component C4 is not involved in renal IRI, given that C4-deficient mice are not protected (21). These findings from MBL- and C4-defiicient mice have been a discrepancy for many years and suggest that other effector functions of MBL besides activation of the lectin pathway of complement might exist. Recently however, it was demonstrated that MBL is able to bypass C2 and C4 and directly activates C3. In different IRI models in mice it was shown that MBL/MASP-1 complexes directly might activate C3 in the absence of C2 and C4 (54), though this was not shown for renal IRI. In the mouse kidney, interaction of MBL with highly glycosylated meprins expressed and secreted by mouse PTEC has been suggested to be the initial step for MBL binding and subsequent complement activation (55), as

these meprins *in vitro* are able to bind MBL and activate complement. Although MBL/MASP-1 complexes are able to bypass C2 and C4 (56), its contribution to the degree of complement activation *in vitro* has been shown to be modest. Since MBL is a highly versatile protein, we cannot exclude that other effectors functions of MBL besides activation of the lectin pathway of complement might play a role in renal IRI.

9.3.1 MBL-mediated tubular injury

In chapter 5 we describe a novel role for MBL in the pathogenesis of renal IRI. Remarkably, this novel role was completely independent of complement activation. In a rat model of renal IRI, we demonstrated that therapeutic inhibition of MBL is protective against renal IRI and prevents kidney dysfunction, tubular damage, neutrophil and macrophage accumulation, and expression of proinflammatory cytokines and chemokines. Following reperfusion, vascular leakage resulted in extravasation of circulation-derived MBL in the interstitial space. Subsequent exposure of tubular epithelial cells to MBL resulted in internalization of MBL followed by the rapid induction of tubular epithelial cell death. This MBL-mediated tubular injury was completely independent of complement activation since inhibition of C3 or C5 was not protective against renal IRI. MBL-mediated cell death preceded complement activation, strongly suggesting that exposure of epithelial cells to MBL immediately following reperfusion is the primary culprit of tubular injury, and not the lytic C5b-9 complex.

Several studies, mainly performed in mice, have shown an important role for complement in the induction of renal IRI. A study using C3, C5 and C6 deficient mice showed a predominant role for C5b-9 in renal IRI (21), and also inhibition of C5 with monoclonal antibodies was protective (57). In contrast to these data, others have shown that gene knock-out (58), inhibition (58) or depletion of C3 (59) was not protective against renal IRI. In our rat IRI model, deposition of C3 and C5b-9 following reperfusion was observed, although this was in a relatively late phase (24 hours onwards). A similar late kinetics of C5b-9 deposition has also been shown in mouse IRI (57), although this mouse model was characterized by an early increase in deposition of C3 (2 hours). We did not observe early C3 deposition, indicating possible differences in kinetics and pathogenesis of renal IRI among species. Differences in organ size and metabolism between species could play a role in the observed differences, given that in mice clamping times of usually 20-25 minutes are needed to induce renal injury, while in rats 45-60 minutes of clamping time is needed to induce substantial IRI. Also, difference in relative strength of complement activation pathways could be of influence. In this respect, it has been shown that serum complement activity even among

rat strains are varying (60), explaining why in certain rat strains complement activation might be more involved. Indeed, the rat strain we used (Lewis), has a relatively low serum complement activity.
Despite the fact that complement activation was not involved in the induction of renal IRI in our rat model, we did find a pivotal role for MBL in the pathogenesis of renal IRI, since transient inhibition of MBL in the early phase following reperfusion was completely protective. It is tempting to speculate that these mechanisms might contribute to a previous observation that genetically determined high levels of MBL are an important risk factor for renal graft loss following clinical kidney transplantation (52;61).
In human intestinal IRI, an association of MBL null alleles with preserved epithelial cell integrity was shown (62). Interestingly, also here no signs of complement activation were observed, suggesting that also in intestinal IRI, MBL might have a cytotoxic effect on epithelial cells. Moreover, MBL has been shown to bear anti-tumor activity to colorectal carcinoma cells, which has been termed MBL-dependent cell-mediated cytotoxicity (63) .
Exposure of tubular epithelial cells to circulation-derived MBL following renal I/R was shown to mediate tubular injury. In normal physiologic conditions, these cells are not exposed to MBL (from the basolateral surface). However, during I/R there is an increased vessel destabilization, endothelial injury and leakage of plasma components in the interstitial compartment. In chapter 8 we demonstrated the presence of such vessel destabilization and loss of endothelial integrity shortly after I/R which was associated by a disturbance in the angiopoietin-1 and -2 balance. This condition enables MBL to diffuse from circulation and reach the epithelial cells from the basolateral side. A therapeutic approach aimed to stabilize endothelial integrity might therefore be successful in several ways. Firstly, leakage of MBL from the vasculature is reduced, thereby preventing MBL to interact with tubular cells. Secondly, a stable endothelium prevents formation of edema and will consolidate the local blood flow. Thirdly, leukocyte interaction and infiltration might be reduced. Therefore, next to blocking MBL following renal I/R, a therapy targeted towards endothelial stabilization might be promising as well.

9.3.2 MBL and TLR signaling

Stressed or dying cells release damage associated molecular pattern molecules (DAMPs), which can signal through pattern recognition receptors (PRR), a class of innate immune response-expressed proteins that respond to pathogen-associated molecular patterns (PAMPs) and DAMPs. A subfamily of these membrane-associated PRR are the Toll-like receptors (TLRs), which sense PAMPs or DAMPs extracellularly or in endosomes and may link innate and

adaptive immune responses. TLRs are present on a variety of cell types including leukocytes, dendritic cells as well as epithelial and endothelial cells. Especially TLR-2 and 4 are highly expressed on PTEC. Interestingly, mice deficient for TLR-2, and -4 are protected against renal IRI (64;65) as shown by a reduction in kidney dysfunction, tubular injury and proinflammatory cytokines, and a concomitant decrease in infiltration by leukocytes. TLR4 signaling through the MyD88-dependent pathway is required for the full development of kidney IRI (65), as both TLR4 and MyD88 deficient mice were protected. It is known that there is a powerful crosstalk between complement and TLR activation in renal IRI (66). Recent studies have shown that there is tight and direct collaboration between MBL and other host defense pathways and receptors to both specify and amplify the immune response following TLR signaling. This is illustrated by the observation that MBL enhances TLR-2 and -6 signaling (67) and by a study showing a tight interaction of MBL with TLR-4 (68). Several endogenous ligands for TLRs like high-mobility group protein B1, hyaluronan and biglycan have been implicated in the pathophysiology of renal IRI (69-74), of which biglycan have been described to interact with MBL (75). It is therefore tempting to speculate about a possible role for MBL in TLR signaling following renal IRI, which might contribute to the protective effect observed in MBL-deficient mice or following therapeutic inhibition of MBL.

9.3.3 MBL and coagulation

Upon ligand binding by MBL, the MBL-associated serine proteases have been shown to not only cleave complement components, but also coagulation factors. For instance, MBL/MASP-1 has been shown to exert thrombin-like activity, to interact with plasma clot formation on different levels and to drive the formation of cross-linked fibrinogen (36;38;39) in a murine model of occlusive thrombosis. MASP-1 is therefore a significant contributor to coagulation and plays a key role in thrombus formation. Also MASP-2 is capable of promoting fibrinogen turnover and generation of a fibrin clot by cleavage of prothrombin, generating active thrombin (37). In addition, MBL is able to bind to fibrinogen and fibrin thereby augmenting clot formation by additional cleavage and activation of fibrinogen and prothrombin (37;76). Several studies have pointed out the relationship between IRI and the coagulation system. Treatment with Antithrombin III (77), tissue factor pathway inhibitor (78) or soluble thrombomodulin (79) following reperfusion in rats reduced renal dysfunction, tubular injury, microvascular leukocyte rolling and attachment and endothelial permeability, indicating that altered coagulation contribute to the pathogenesis of ischemic renal injury. De Vries et al (32) recently observed a release of prothrombin fragment 1 and 2, which are formed when prothrombin is converted to thrombin, shortly after living

donor transplantation. The rapid activation of coagulation by the transplanted kidney may lead to the generation of fibrin, which might damage the graft by clot formation in the microvasculature (32;80). The parallel expression of activation products of the coagulation, fibrinolysis, and complement systems has long been observed in both clinical and experimental settings. In man, enhanced thrombin generation after reperfusion of deceased donor kidneys might be a part of the explanation of the poorer outcome of transplantation observed in deceased compared to living donor kidneys (81). High pretransplantation levels of MBL, which is associated with patient and graft survival after kidney transplantation, might predispose to enhanced microvascular thrombosis through MBL/MASP-mediated activation of coagulation. Therefore, thrombus formation and coagulopathy might be an additional way in which MBL is involved in renal IRI.

9.3.4 Mechanism of MBL-mediated tubular injury

PTEC that encounter nutrient and oxygen deprivation during ischemia use various adaptive responses to maintain homeostasis and survival, including the initiation of macroautophagy and the activation of the ER stress or unfolded protein response (UPR). These adaptive responses decrease energy expenditure, increase nutrient availability and promote cell survival. However, these biological processes also lead to the generation of proinflammatory cytokines and chemokines, indicating that stressed tissues generate alarm signals that produce an inflammatory microenvironment (82). In chapter 6, the thesis reports a possible mechanism by which MBL mediates direct 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. We demonstrated that inhibition of MBL *in vivo* protected tubular cells from loss of vital GRP78 and subsequent tubular injury. GRP78 is a constitutively expressed molecular chaperone, however expression is enhanced under a variety of stressful conditions including hypoxia, glucose deprivation, alterations in intracellular calcium and oxidative stress (83), events that take place during renal I/R. Induction of GRP78 is critical for maintaining viability of cells that are subjected to such stresses and loss of GRP78 would therefore be detrimental (84;85). We demonstrated that renal I/R is accompanied by ER-stress and loss of GRP78 in the cortico-medullary region of kidney, the region that is mostly affected. Importantly, therapeutic inhibition of MBL protected tubular cells located in this region from loss of vital GRP78 and consequent tubular injury, indicating that MBL might be injurious to tubular cells by interfering with GRP78. Expression of proinflammatory IL-6, a consequence of GRP78 degradation (86) and ER-stress (82;87), was also significantly reduced by inhibition of MBL following reperfusion.

Internalization of circulation-derived MBL *in vivo* was shown to be the process

by which tubular epithelial cells following renal I/R might be injured (41). By exposing tubular cells *in vitro* to MBL, we demonstrated that basolateral internalization of MBL by tubular cells induced a potent ER-stress response which was accompanied by an excessive ATP production, a subsequent loss of the mitochondrial membrane potential, production of mitochondrial superoxide followed by induction of cell death. Internalized MBL colocalized with mitochondria and GRP78. Although GRP78 is mostly known from its function as molecular chaperone in the ER, GRP78 has also been shown to be present, although in low amounts, on mitochondria, in the cytoplasm, on the cell membrane and as a secreted form (88-93). Membrane-bound GRP78 might be a possible candidate as a cellular receptor by which MBL is internalized.

It has been shown that ligation of cell-surface GRP78 on cancer cells causes release of calcium from ER stores mediated via phospholipase C, thereby increasing cytosolic calcium (94). In line with this, it is known that especially tumor cells have increased levels of GRP78 on the cell surface. Importantly, is has been shown that MBL bears antitumor effects, which has been termed MBL-dependent cell-mediated cytotoxicity (63). Since MBL is able to interact with GRP78, we speculate that, next to killing of tumor cells, the cytotoxic effect of MBL on tubular cells might be mediated by signaling through cell surface GRP78. Under physiologic conditions GRP78 is hardly present on normal cells, however induction of ER-stress has been shown to promote GRP78 localization on the surface of kidney cells (93). ER-stress induced by glucose deprivation during renal ischemia might be the initial trigger for GRP78 to traffic to the basolateral membrane of the tubular cells. Especially tubular cells in the cortico-medullary border are subjected to low oxygen and glucose levels during I/R, making these cells particular sensitive to ER-stress. Vascular leakage following reperfusion enables circulation-derived MBL to enter the interstium and there might bind to GRP78 on the basolateral surface of tubular cells. Signaling to GRP78 might then have detrimental effects. Next to signaling, internalization of MBL might also be mediated by GRP78, since evidence has emerged that GRP78 also serves as a receptor for viral entry into host cells (95). Since the ligand for MBL on tubular cells has not been found thus far, these speculations still need to be confirmed.

These findings until now suggest that initially ER-stress is induced following MBL exposure, followed by disturbances in mitochondrial homeostasis. Release of calcium from ER stores might be the main cause of MBL-mediated ER-stress. An increase in cytosolic calcium in tubular cells then would lead to enhanced calcium influx into mitochondria, disrupting mitochondrial metabolism eventually leading to tubular cell death.

9.3.5 Therapeutic inhibition of MBL

Upon reperfusion of the ischemic kidney, MBL from circulation leaks into the interstitium where it is internalized by tubular cells and induces tubular injury. Unfortunately, to date no anti-MBL therapy or other treatments for renal IRI in the clinic exists. Recently however, several clinical possibilities of pharmacologic inhibition of complement, including C1 inhibitor (C1INH) and Eculizumab (anti-C5 monoclonal antibody) have become available (31).

C1INH, a physiologic serine protease inhibitor belonging to the serpin superfamily, acts as a major inhibitor of complement system activation and prevents uncontrolled complement activation. C1INH might be a possible therapeutic candidate to prevent renal IRI given that C1INH regulates vascular permeability (96) and might prevent leakage of MBL in the interstitial compartment following reperfusion. Secondly, C1INH targets the MASPs, which might be involved in the cytotoxic effect of MBL on tubular cells. Thirdly, complement that is activated at a later stage following reperfusion leading to exacerbation of tissue injury might be inhibited. Recombinant human C1INH, which recently has been registered for treatment of hereditary angioedema, is differentially glycosylated (97;98) with high oligo-mannose structures and therefore might include an additional inhibitory effect towards MBL recognizing mannose residues. We explored the therapeutic application of rhC1INH (chapter 7) in a rat model of renal IRI and studied whether rhC1INH is able to attenuated MBL-mediated tubular injury. We demonstrated that rhC1INH is functional active in rat serum and blocks both classical and lectin pathway activation *in vitro*. However, the therapeutic administration of rhC1INH *in vivo* did not attenuate renal dysfunction, tubular injury, inflammatory cell influx and complement activation following reperfusion in rats. Kinetic studies revealed that rhC1INH was cleared from circulation within 2 hours, complement activity was only shortly reduced and circulating MBL levels were unaffected. In addition, we demonstrated that rhC1INH was not able to block the MBL-mediated cytotoxicity towards human tubular cells *in vitro*.

Recently, a clinical trial for the prevention and treatment of reperfusion injury in kidney transplantation has been started (NCT01756508). In this trial, C5 is targeted using the humanized monoclonal antibody (Eculizumab). This antibody binds to C5 and prevents cleavage of C5 and formation of C5b-9 and C5a. Our studies in rats and human however do not support a key role for C5 in renal IRI, given that inhibition of C5 using an identical antibody targeting rat C5 was not protective a no release of human sC5b-9 and C5a or local C5b-9 deposition could be detected early after clinical reperfusion. Importantly, our data demonstrate that therapeutic targeting of MBL following reperfusion might be very promising. Currently, a mouse monoclonal blocking antibody to human MBL (3F8) is available and in the near future will be humanized for therapeutic application in humans.

When clinically available, short interference with MBL using this antibody may have important therapeutical implications. In our rat model, we demonstrated that a single injection of anti-MBL antibody shortly before reperfusion provides an almost complete protection against renal IRI. In the clinical setting of kidney transplantation, a short treatment of the recipient with an anti-MBL antibody therefore could significantly alter the extent of kidney damage following renal IRI.

9.4 CONCLUSIONS

Although the main function of complement is to clear pathogens and maintaining homeostasis by clearing apoptotic cells and cellular debris, complement activation might also contribute to renal IRI by exacerbating local inflammation and tubular injury through release of C5a and formation of C5b-9. Studies mainly performed in mice have demonstrated an important role for the alternative pathway. Nevertheless, in this thesis we show that human hypoxic PTEC predominantly activates the classical pathway of complement, demonstrating important species-specific differences in complement activation. In addition we showed that early following reperfusion in human there is only a very transient activation of complement in circulation, which is virtually absent at the tissue level. We also demonstrated that it is not reliable to assess sC5b-9 in urine, given that spontaneous C5b-9 generation can occur, independent of a renal contribution. From these findings and kinetic data obtained from our rat IRI model, we conclude that complement activation following reperfusion is merely a secondary process activated by injured and apoptotic tubular cells in a relatively late phase following reperfusion (Fig 1). In contrast, early after reperfusion circulation-derived MBL, independent of complement activation, exerts cytotoxic effect to tubular cells mediating initial tubular injury. Although not shown in this thesis, MBL might also be involved in potentiating TLR signaling, which have been implicated in the pathophysiology of renal IRI. Furthermore, a crosstalk between MBL and coagulation might be involved, facilitating coagulation and clot formation in the renal microvasculature and contributing to the coagulopathy in renal IRI.

In short, MBL is a highly versatile protein which, in addition to potent complement activation, also exerts direct cytotoxic effect, amplifies TLR signaling and promotes coagulation. In conclusion, this thesis documents the important role of MBL in the early pathogenesis of renal IRI and shows that complement activation is a relatively late process in the pathophysiology of renal IRI. Treatment with rhC1INH, or targeting C3 and C5 was not protective against renal IRI. In contrast,

interfering with MBL was highly effective in reducing renal IRI, indicating that MBL has a pivotal role in the pathophysiology of renal IRI. We demonstrate a crucial role for MBL in renal IRI and identify MBL as a novel therapeutic target in kidney transplantation. We therefore suggest that a short therapeutic intervention at the level of MBL might significantly alter the extent of kidney damage following renal I/R. The development and application of a humanized anti-MBL antibody might be a promising step forward in the treatment of renal IRI following kidney transplantation.

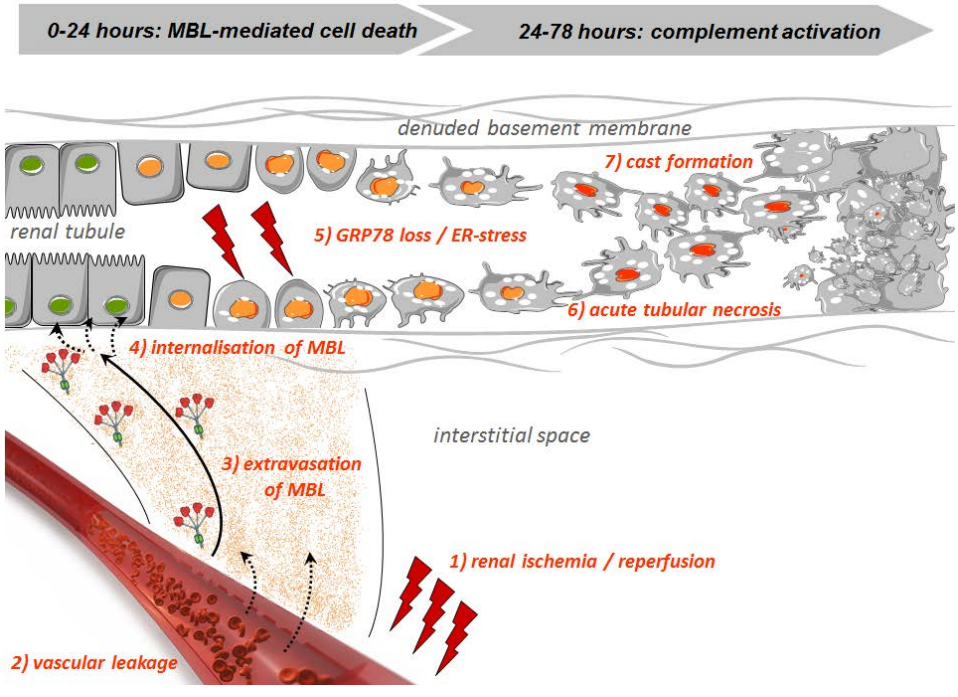

Figure 1. Role of complement and MBL in renal ischemia/reperfusion injury. Renal I/R is associated with a dysbalance of angiopoietins and vessel destabilization (**chapter 8**), resulting in vascular leakage, edema and extravasation of MBL and other complement molecules like C1q in the interstitial space (**chapter 5**). Basolateral exposure of tubular epithelial cells to MBL results in binding and internalization of MBL, which is accompanied by loss of ER-stress sensor GRP78, induction of endoplasmic reticulum-stress (chapter 6) and subsequent tubular cell death within several hours of reperfusion (**chapter 6**). Injury to the tubular cells lead to activation of the complement system in the later phase of reperfusion and in human is mediated by natural IgM and C1q (**chapter 2**). In circulation or at the tissue level, no complement activation products could be detected early after reperfusion (**chapter 3**), while in the urine complement measurement is not reliable (**chapter 4**). Therapeutic targeting of MBL was highly protective against renal IRI, whereas blocking of complement activation at the level of C3 and C5 (**chapter 5, 7**) was not, indicating that MBL play a pivotal role in renal IRI, independent of complement activation.

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CHAPTER 10

Nederlandse samenvatting
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NEDERLANDSE SAMENVATTING

Ischemie/reperfusie schade

Tussen de uitname en transplantatie van een donornier is er geen doorbloeding van het orgaan. Hierdoor ontstaat een tekort aan zuurstof, glucose en andere voedingstoffen en een opstapeling van afvalstoffen zoals koolstofdioxide in de nier. Deze toestand in het orgaan wordt ischemie genoemd. Door het tekort aan zuurstof tijdens ischemie kan de nier geen energie produceren en ontstaat er weefselschade die te vergelijken is met bijvoorbeeld een hersen- of hartinfarct. Alle levensprocessen zoals bewegen, maar ook bijvoorbeeld het stabiel en gezond houden van onze cellen, kosten energie. Deze energie wordt verkregen door de verbranding van suikers en vetten waarbij ook zuurstof benodigd is. Iedere cel in ons lichaam bevat een soort energiefabriekjes, mitochondriën genaamd, waarin met behulp van zuurstof, suikers en vetten verbrand worden. De energie die daaruit vrijkomt, wordt opgeslagen in de chemische vorm van adenosine trifosfaat (ATP). ATP is dus de chemische drager van energie in al onze cellen. We kunnen ATP vergelijken met de stroom die wordt opgewekt in een elektriciteitscentrale wanneer kolen en gas (net zoals suikers en vetten in onze cellen) verbrand worden. Zonder ATP in onze cellen kunnen wij niet leven. Tijdens de ischemie is er niet genoeg zuurstof en brandstof om ATP te produceren en ontstaat er weefselschade in de nier. We noemen deze schade ischemieschade.
Het herstellen van de bloedtoevoer na een niertransplantatie wordt reperfusie genoemd. Bij de reperfusie van een ischemisch orgaan worden schadelijke afvalstoffen afgevoerd en zuurstof, glucose en andere belangrijke stoffen aangevoerd die nodig zijn om het weefsel te herstellen. Het lijkt tegenstrijdig, maar het is bekend dat de reperfusie van een ischemisch orgaan echter tot nog veel meer weefselschade leidt. De schade na de reperfusie noemen we daarom ischemie/reperfusie schade (I/R schade).
Het is aangetoond dat I/R schade kort na de transplantatie een erg ongunstige invloed heeft op de functie van de nier op de lange termijn. Het is daarom dus erg belangrijk om deze I/R schade als gevolg van de onderbroken bloedtoevoer tijdens de niertransplantatie te voorkomen of zoveel mogelijk te beperken. Hierover gaat het onderzoek beschreven in dit proefschrift.
De oorzaak van I/R schade is nog onvoldoende duidelijk en er is daarom op dit moment nog geen gerichte behandeling ontwikkeld om I/R schade te beperken. Bij het ontstaan van I/R schade speelt ons aangeboren afweersysteem een rol. Het complementsysteem waarover dit proefschrift vooral gaat, is een onderdeel hiervan. Dit complementsysteem doodt indringende bacteriën via een serie activerende factoren in het bloed. Voordat hierop dieper ingegaan zal worden, is enige uitleg van het immuunsysteem en de fysiologie van de nier nodig.

Fysiologie van de nier

Om ons bloed te filteren, bevatten onze nieren ongeveer 1 miljoen nierfiltertjes (glomeruli) waar het bloed doorheen stroomt. De glomerulus is een soort klein vergietje van 0,2 mm grootte dat kleine eiwitten, zouten, water en afvalstoffen uit het bloed doorlaat. Grote eiwitten en cellen passen echter niet door de gaatjes en verlaten de glomerulus weer via een afvoerend bloedvat. Het filtraat dat uit de glomerulus komt, wordt opgevangen in een nierbuisje (tubulus). Deze nierbuisjes (tubuli), waarvan er dus ook 1 miljoen zijn omdat ze verbonden zijn met de glomeruli, monden uit in het nierbekken waar de urine verzameld wordt voordat het naar de blaas toestroomt.

In deze tubuli vindt een ontzettend belangrijk proces plaats, dat we tubulaire reabsorptie noemen. Dagelijks wordt door de nieren namelijk 180 liter filtraat (voorurine) geproduceerd, waarvan slechts 1-2 liter wordt uitgescheiden als urine. De voorurine, die belangrijke eiwitten, zouten en suikers bevat, wordt grotendeels weer opgenomen in het bloed via een laagje cellen dat zich aan de binnenkant van de tubuli bevindt. We noemen deze cellen tubulus epitheelcellen en het proces van opname heet dus tubulaire reabsorptie. Zonder dit proces zouden we binnen enkele minuten al onze eiwitten, water, zouten en suikers uit ons bloed verliezen. Het proces van tubulaire reabsorptie kost erg veel energie (ATP). Daarom bevatten de tubulus epitheelcellen veel mitochondriën en gebruiken ze veel zuurstof uit het bloed. Om de opgenomen stoffen terug te transporteren naar het bloed, zitten er veel kleine bloedvaatjes om de tubuli heen. Deze bloedvaatjes nemen de belangrijke stoffen via de tubulus epitheelcellen uit de urine weer op in bloed. Een nadeel hiervan is dat ook veel zuurstof dat eigenlijk bestemd is voor de tubulus epitheelcellen, weer opgenomen wordt in het bloed, terwijl de zuurstofbehoefte in de epitheelcellen juist groot is. Normaal gesproken is dit precies in balans. Echter bij een niertransplantatie, wanneer de bloed- en zuurstoftoevoer stopt, gaat het mis. De tubulus epitheelcellen die veel zuurstof nodig hebben om ATP te maken voor tubulaire reabsorptie, raken gestrest en gaan dood wanneer de bloedtoevoer te lang stopt. We noemen dit acute tubulus necrose (ATN). Bij ATN als gevolg van een ATP-tekort raken de tubulus cellen los van de tubuluswand waar ze op groeien en komen in de holte (lumen) van de nierbuisjes terecht. Hier gaan de cellen dood (necrose) en vallen uit elkaar in kleine deeltjes. Samen met gefilterde eiwitten vormen deze deeltjes grote klompen celmateriaal (casts), waardoor de nierbuisjes verstopt raken en de nierfunctie stopt. Vooral bij nieren die afkomstig zijn van overleden donoren en dus een lange ischemietijd hebben, is de tubulus necrose en I/R-schade groot. Het duurt daarom ook vaak enkele dagen voordat deze getransplanteerde nieren weer enigszins functioneren. In deze dagen is de patiënt aangewezen op nierdialyse.

Het complementsysteem

De belangrijkste functies van ons afweer- of immuunsysteem is om ons te beschermen tegen ziekteverwekkers (pathogenen) zoals bacteriën en virussen. De afweercellen in ons bloed die hierbij betrokken zijn, noemen we witte bloedcellen (leukocyten). Deze cellen worden geholpen door een groep eiwitten (proteïnen) in ons bloed die ook deel uit maken van ons afweersysteem. Eiwitten zijn biologische moleculen en zijn essentieel voor organismen. Ze bestaan uit lange ketens van aminozuren. Eiwitten hebben heel veel verschillende functies. In onze cellen zijn ze betrokken bij chemische omzettingen (enzymen), het in stand houden van de structuur van cellen (cytoskelet), transport van stoffen in, uit en binnenin de cel en voor communicatie tussen cellen (bijvoorbeeld hormonen).

In ons bloed bevindt zich een groep eiwitten die naast de witte bloedcellen betrokken is bij ons immuunsysteem. Deze eiwitten heten complementfactoren en samen vormen deze complementfactoren het zogenaamde complementsysteem. Het complementsysteem speelt samen met de witte bloedcellen en antistoffen (ook een groep eiwitten) een zeer belangrijke rol bij de afweer tegen ziekteverwekkers. Het complementsysteem is een systeem van factoren in het bloed dat verantwoordelijk is voor het lek maken (lyseren) van met antilichamen bedekte bacteriën en virussen. Deze complementfactoren maken samen gaatjes (poriën) in de wand van de bacterie waardoor deze lyseert en dood gaat.

Onder normale omstandigheden verkeert het complementsysteem in een niet-geactiveerde staat. Activatie en daarmee het ontstaan van biologische activiteit kunnen plaatsvinden na interactie van complementfactoren met bijvoorbeeld antistoffen die gebonden zijn aan een bacterie of rechtstreeks met allerlei bacteriën en virussen zonder tussenkomst van antilichamen.

Het complementsysteem werkt direct vanaf de geboorte en hoeft daarom niet geleerd te worden hoe om te gaan met ziekteverwekkers. Het maakt daarom deel uit van het zogenaamde aangeboren (innate) immuunsysteem. Wanneer een ziekteverwekker zoals een bacterie ons lichaam binnendringt, wordt het allereerst aangevallen door het complementsysteem. Het complementsysteem werkt als een soort kettingreactie. Er zijn allereerst enkele eiwitten in het complementsysteem die de ziekteverwekkers herkennen en hieraan binden. Eén van deze “herkennende” complementfactoren, waar dit promotieonderzoek ook overgaat, is Mannan-binding lectin (MBL). Deze complementfactor herkent bepaalde suikermoleculen (mannan en mannose) die voorkomen op ziekteverwekkers, maar ook op onze eigen cellen wanneer deze dood gaan. Wanneer MBL bindt aan een dergelijk suikermolecuul op bijvoorbeeld een bacterie, verandert de vorm van het MBL-eiwit en wordt het actief. Vervolgens ontstaat een kettingreactie waarbij verschillende complementfactoren elkaar

activeren en gebonden worden op het oppervlak van de ziekteverwekker. We noemen dit de lectine route activatie van het complementsysteem. Andere "herkennende" complementfactoren zijn C1q en properdine. Deze complementfactoren activeren weer een andere route of kettingreactie van het complementsysteem, namelijk de klassieke route door C1q en de alternatieve route door properdine. Uiteindelijk komen alle drie de routes uit bij het centrale molecuul van het complementsysteem, namelijk complement factor C3.
Activatie van C3 leidt tot splitsing van het C3 molecuul in C3a en C3b. Het C3b wordt stevig (covalent) gebonden op het oppervlak van de bacterie. Activatie van het complementsysteem leidt dus altijd tot een laagje C3b dat de ziekteverwekker als het ware bedekt. Op deze manier vormt het complementsysteem een soort C3b vlaggetjes op de ziekteverwekker, waardoor deze als het ware gemarkeerd (geopsoniseerd) wordt om opgeruimd te worden. De witte bloedcellen hebben en soort antennes (receptoren) op hun oppervlak die de C3b vlaggetjes herkennen en de gemarkeerde bacterie vervolgens kunnen opeten (fagocyteren).
Daarnaast kunnen andere complementfactoren (C5, C6, C7, C8, C9) ook op volgorde geactiveerd raken via C3b. Hierbij wordt eerst het C5 molecuul gesplitst in C5a en C5b. Vervolgens binden de factoren C6-C9 aan het gesplitste C5b en maken samen een gaatje (porie) in de bacterie, waarna deze lyseert. Het C5b-C9 complex wordt daarom ook wel het Membrane-Attack-Complex (MAC) genoemd. Activatie van C5 tot C9 wordt de terminale route van het complementsysteem genoemd.
Naast het opsoniseren met C3b (waardoor de ziekteverwekker gemarkeerd wordt voor opruiming) en het lyseren door het C5b-9 complex (waardoor de ziekteverwekker lek raakt), heeft het complementsysteem nog een derde functie. Bij de splitsing van C3 en C5 worden de splitsingsfactoren C3a en C5a gevormd. Deze factoren worden ook wel anaphylatoxines genoemd en trekken witte bloedcellen aan naar de plek van de ontsteking. Daarnaast activeren C3a en C5a ontstekingscellen en leiden ze tot contractie van gladde spiercellen, het lekken van de bloedvaten en het vrijmaken van histamine en ontstekingseiwitten. Het gevolg hiervan is dat een ontsteking (inflammatie) ontstaat en het weefsel rood en warm wordt. Onze eigen cellen zijn beschermd tegen activatie van het complement door zogenaamde complementregulatoren. Deze regulatoren voorkomen dat onze cellen bedekt raken met een laagje C3b of lek raken door het poriecomplex C5b-9 en zodoende een ontsteking veroorzaken.
Zoals al eerder gezegd, kunnen ook dode cellen in ons lichaam het complementsysteem activeren. Elke dag gaan er meer dan 100 miljard van onze cellen (o.a. door veroudering) dood. Tijdens het dood gaan verliezen deze lichaamseigen cellen ook hun complementregulatoren. Hierdoor activeren ook deze cellen het complementsysteem en raken bedekt met C3 om effectief

opgeruimd te worden. Zolang het slechts bij activatie van C3 blijft en er geen C5a en C5b-9 (MAC) gevormd wordt, verloopt dit opruimingsproces geruisloos zonder ontsteking. Personen die bijvoorbeeld C1q missen, ontwikkelen vaak auto-immuun ziekten die samenhangen met het verkeerd opruimen van de enorme hoeveelheid (eigen) dode cellen. Dit laat dus zien hoe belangrijk het complementsysteem ook bij het opruimen van dode cellen is.

Complement in nier ischemie/reperfusie schade

Het complementsysteem heeft dus een belangrijke en gunstige rol in ons immunsysteem. Echter bij bepaalde aandoeningen, zoals I/R in de nier gaat het mis. Door het ATP-tekort tijdens de ischemie en door de reperfusie verliezen de overlevende tubulus epitheelcellen in de nier hun beschermende complementregulatoren. Hierdoor raken deze cellen erg vatbaar voor complementactivatie. De cellen die tijdens de ischemie door het ATP-tekort al dood zijn gegaan, hebben ook hun complementregulatoren verloren. Hierdoor ontstaat in de dagen na de I/R een enorme activatie van het complementsysteem. Een studie in muizen heeft laten zien dat door het C5b-9 complex veel tubulus epitheelcellen lek raken en dood gaan. Daarnaast worden door de vorming van C3a en C5a ook veel ontstekingscellen aangetrokken, gaan de bloedvaatjes openstaan en ontstaat er vochtophoping in de nier (oedeem). Door dit oedeem worden de bloedvaatjes dichtgedrukt en kan er opnieuw ischemie optreden met als gevolg nog meer nierschade. Muizen waarin bepaalde complementfactoren, zoals C3 en MBL genetisch uitgeschakeld zijn, ontwikkelen veel minder schade na reperfusie van de ischemische nier. Ook muizen waarin het C5a therapeutisch weggevangen wordt, ontwikkelen minder I/R schade. Deze studies laten zien dat in muizen het complementsysteem in ieder geval een belangrijke rol speelt in I/R schade.

Proefschrift samenvatting

Het doel van het onderzoek beschreven in dit proefschrift was het bestuderen van de rol van het complementsysteem in I/R schade in de nier. Zoals eerder gezegd, bevat het complementsysteem verschillende routes van activatie; de klassieke route via C1q, de lectine route via MBL en de alternatieve route via properdine. Om een therapie te ontwikkelen waarbij het complementsysteem geremd kan worden, is het van belang om te weten welke specifieke routes van het complementsysteem geactiveerd worden na I/R in de nier. Daarnaast is er veel onderzoek gedaan in muizen, maar is er nauwelijks iets bekend over de rol van complement in de mens na I/R van de nier.

In **hoofdstuk 2** hebben we daarom onderzocht welke route van het complementsysteem in werking wordt gezet door muis en menselijke niercellen

na I/R. Vooral de tubulus epitheelcellen in de nier raken beschadigd na I/R. We hebben daarom deze cellen geïsoleerd uit nieren van muizen en mensen, en deze cellen gekweekt in aan- of afwezigheid van zuurstof. Op deze manier simuleerden we het proces van ischemie tijdens een niertransplantatie. Na een aantal uur werden de ischemische niercellen weer van zuurstof voorzien en blootgesteld aan muis of menselijk bloed (reperfusie). Dit bloed bevat de complementfactoren die vervolgens geactiveerd konden worden. Wat we vervolgens waarnamen, was dat de gekweekte muis epitheelcellen na I/R de alternatieve route van het complementsysteem activeerden. We bevestigden daarmee publicaties van anderen betreffende dit onderzoek uitgevoerd in muizen. Verrassend was het echter dat de menselijke tubulus epitheelcellen deze alternatieve complementroute helemaal niet activeerden, maar wel herkend werden door IgM antilichamen en een andere complementfactor, namelijk C1q. Door de binding van IgM en C1q aan deze cellen werd vervolgens de klassieke route van complement geactiveerd. Deze studie laat dus duidelijk zien dat er verschillen zijn tussen muis en menselijke tubulus epitheelcellen en het complementsysteem. Bij het ontwikkelen van een therapie gericht tegen een bepaalde route van het complementsysteem dient hier zeker rekening mee gehouden te worden.

In **hoofdstuk 3** werd onderzocht in welke mate complementactivatie voorkomt na een niertransplantatie in de mens. Hoewel er veel bekend is uit onderzoek in muizen, zijn er nauwelijks onderzoekgegevens beschikbaar van I/R schade na niertransplantatie in de mens. De reden hiervoor is dat I/R schade kort na een niertransplantatie optreedt en het tot op heden niet mogelijk was nierweefsel van patiënten en bloed uit de getransplanteerde nier kort na transplantatie te verkrijgen. We hebben daarom gebruikt gemaakt van een geheel nieuwe methode die recent ontwikkeld is op de afdeling Heelkunde in Leiden. Bij deze methode wordt via een kleine catheter bloed afgenomen dat direct afkomstig is uit de getransplanteerde nier. Hierdoor waren we in staat om daadwerkelijk te meten hoeveel complementactivatie er in de getransplanteerde nier plaatsvindt, terwijl eerder alleen systemische metingen (niet in de nier maar in het lichaam) mogelijk waren. Ook was het mogelijk om zowel voor, als 45 minuten na de niertransplantie een nierbiopt te nemen, zodat ook in het weefsel onderzocht kon worden in welke mate er activatie van complement plaatsvond op de epitheelcellen. Deze studie vond plaats in drie groepen patiënten. De eerste groep patiënten ontving een nier van een levende donor, de tweede groep van een hersendode donor en de derde groep van een overleden donor. Vooral de donornieren uit de derde groep zijn in relatief slechte staat vanwege de bloedcirculatie die al gedurende langere tijd stilstaat.

In het weefsel en in het bloed werd vervolgens de complementactivatie bepaald

door het aankleuren en meten van C5b-9, het terminale eiwitcomplex van het complementsysteem. Verrassend genoeg was er in alle drie de groepen op het weefsel totaal geen complementactivatie aantoonbaar 45 minuten na de niertransplantatie. Alleen in de patiënten die een nier ontvingen van een overleden donor, was enkele seconden na de reperfusie C5b-9 aantoonbaar in het bloed, dit was echter zeer tijdelijk. Concluderend laat deze studie zien dat in de mens vroeg na transplantatie nauwelijks complementactivatie plaatsvindt in circulatie, en dat deze ook volledig afwezig is op het weefsel. Dit suggereert dat activatie van het complementsysteem in de mens een secundair proces is, en niet ten grondslag ligt aan de initiële ischemie/reperfusie schade die optreedt na niertransplantatie.

Naast het aantonen van complementactivatie in het bloed en op het weefsel, is een derde mogelijkheid het meten van complementactivatieproducten (C5b-9) in de urine. Met de studie beschreven in **hoofdstuk 4** werd getracht dit te doen. Doordat de glomeruli bij een niertransplantatie tijdelijk niet goed werken, kunnen kleine maar ook grotere eiwitten uit het bloed, waaronder ook complementfactoren in de (voor)urine terecht komen. Hier kunnen deze complementfactoren in contact komen met de (ischemische) tubulus epitheelcellen en geactiveerd worden. In eerste instantie vonden we inderdaad een duidelijk en significant verband met complementactivatieproducten (C5b-9) en de hoeveelheid eiwit in de urine afkomstig van getransplanteerde nieren. Echter na enkele controle experimenten met urine van gezonde individuen, waaraan bloed (met nog niet geactiveerde complementfactoren) werd toegevoegd, bleek dat complementactivatie in urine ook spontaan kan optreden zonder enige bijdrage vanuit de nier. Dit was tot nog toe niet bekend en uit vervolgonderzoek bleek dat deze complementactivatie ook kan ontstaan door dode epitheelcellen die normaal gesproken ook via de urine worden uitgescheiden. Het bleek dus niet mogelijk een betrouwbare meting van complementactivatie in urine van transplantatiepatiënten te doen, omdat dit beeld vertroebeld wordt door de spontane activatie die kan optreden. Uit eerdere klinische studies in niertransplantatiepatiënten is gebleken dat patiënten met hoge MBL waarden in het bloed meer afstoting en een slechtere transplantaatoverleving hebben op langere termijn. MBL is het eiwit dat suikergroepen op cellen herkent en vervolgens de lectine route van complement activeert. In **hoofdstuk 5** onderzochten we daarom de rol van MBL in I/R schade in de nier. Ook bestudeerden we de complementfactoren C3 en C5 die door MBL geactiveerd worden. We gebruikten hiervoor een I/R model in de rat, waarbij we voor 45 minuten de vaten naar de linker nier afklemden. Op deze manier wordt de I/R schade gesimuleerd die ook ontstaat wanneer een nier wordt uitgenomen bij een niertransplantatie een enige tijd bewaard wordt, voordat hij teruggeplaatst kan worden. Door de andere nier in de rat te verwijderen, waren

we in de staat de nierfunctie van de tijdelijk afgeklemde nier te meten. Na drie dagen werden de ratten opgeofferd en de nier verwijderd voor onderzoek. Als therapie werd vóór het afklemmen van de nier een antilichaam ingespoten dat aan MBL bindt en hierdoor de werking van dit eiwit blokkeert. In twee andere behandelgroepen werden voor het afklemmen, de complementfactoren C3 en C5 in de rat therapeutisch geremd. Vervolgens werd om de 24 uur bloed afgenomen om de nierfunctie te bepalen. Hieruit bleek dat ratten die tijdelijk geen MBL in het bloed hadden, volledig beschermd waren tegen I/R schade als gevolg van het afklemmen. Dit betekent dus inderdaad dat MBL, dat normaal belangrijk is voor de afweer tegen ziekteverwekkers, een zeer nadelige rol heeft in niertransplantatie. Verrassend genoeg waren de ratten waarin C3 en C5 geremd werd, totaal niet beschermd. Omdat MBL bekend staat als een complementeiwit dat C3 uiteindelijk activeert, was deze vinding bijzonder. Dit betekent namelijk dat de nadelige werking van MBL in I/R schade in de nier onafhankelijk is van complementactivatie, iets dat totaal onbekend is. We konden laten zien dat kort na reperfusie, MBL uit de bloedvaatjes lekt en bindt aan de tubulus epitheelcellen in de nier. Vervolgens gaan deze epitheelcellen dood, waarna dan pas het complementsysteem geactiveerd wordt. Complementactivatie vindt dus uiteindelijk wel plaats, maar de initiële schade ontstaat door MBL. Wanneer we gekweekte menselijke tubulus epitheelcellen blootstelden aan (uit bloed gezuiverd) MBL, trad binnen 24 uur celdood op. Hierdoor konden we laten zien dat niet alleen in de rat, maar ook bij mensen blootstelling van de nier aan MBL schadelijk is. Via welke mechanisme MBL de epitheelcellen doodt, wordt nu vervolgens onderzocht.

In **hoofdstuk 6** wordt dit onderzoek naar de cytotoxische (celdodende) werking van MBL op tubulusepitheel beschreven. We laten zien dat MBL bindt en opgenomen wordt in de tubulus epitheelcellen. Vervolgens ontstaat er stress in het deel van de cel, dat verantwoordelijk is voor de productie van eiwitten, het endoplasmatisch reticulum (ER). Als gevolg van deze ER-stress ontstaat er ook een probleem in de mitochondriën van de cellen en komen deze zonder ATP te zitten. In ratten konden we daarnaast laten zien dat het eiwit GRP78 in de tubulus epitheelcellen van de nier binnen enkele uren na reperfusie volledig verdwijnt, waarna deze cellen vervolgens dood gingen. GRP78 is een eiwit in het ER dat ervoor zorgt dat nieuw-geproduceerde eiwitten goed in elkaar gezet en gevouwen worden. Zonder GRP78 ontstaat er een ophoping van niet-functionerende eiwitten in het ER en raken de cellen gestrest (ER-stress) en gaan uiteindelijk dood. Wanneer we nu voor de reperfusie, MBL therapeutisch blokkeerden in deze ratten, bleef het GRP78 ook aanwezig in de cellen en overleefden deze de ischemie/reperfusie. Dit laat dus duidelijk zien dat het wegvangen van MBL voorkomt dat het onmisbare GRP78 in de epitheelcellen

verdwijnt en er ischemie/reperfusie schade ontstaat.
In **hoofdstuk 7** onderzochten we of het medicijn, recombinant menselijk C1 inhibitor (rhC1INH) dat momenteel gebruikt worden in de behandeling van hereditair angio-oedeem, ook effectief is tegen I/R schade in de nier. De hypothese is dat rhC1INH voorkomt dat de vaatjes gaan lekken na I/R waardoor MBL niet bij de epitheelcellen kan komen. Ten tweede remt het enkele complementfactoren en ten derde is er een direct remmend effect op MBL mogelijk, omdat rhC1INH enkele suikergroepen bevat waaraan MBL mogelijk kan binden. rhC1INH werd getest in het I/R model in de rat. Vijf minuten voor het afklemmen van de nier werd het ingespoten. Bloedmonsters werden vervolgens afgenomen om de nierfunctie te bepalen en na 24 uur werd de nier uitgenomen voor analyse. Helaas bleek het rhC1INH niet beschermt tegen I/R schade. Uit vervolgonderzoek bleek dat rhC1INH erg snel verwijderd wordt uit het bloed en daarnaast niet in staat is om het cytotoxische effect van MBL te remmen.
Omdat na I/R, MBL uit de vaatjes lekt en zodoende bij de epitheelcellen kan komen, werd in **hoofdstuk 8** onderzocht hoe de stabiliteit van deze vaatjes gereguleerd wordt. Angiopoetines zijn eiwitten die hierbij nauw betrokken zijn. Bepaalde cellen die de bloedvaten ondersteunen (pericyten) produceren Angiopoetine-1 (Ang-1), dat belangrijk is voor de stabiliteit van het bloedvat. Angiopoetine-2 (Ang-2), dat juist tegenovergesteld werkt en zorgt dat de vaatjes gaan lekken, kan worden geproduceerd door endotheelcellen aan de binnenkant van de bloedvaten. Zolang er meer Ang-1 dan Ang-2, blijven de bloedvaatjes stabiel. Wanneer echter door een niertransplantatie de endotheelcellen gestrest raken, wordt er meer Ang-2 geproduceerd en ontstaat er een verkeerde balans waardoor de bloedvaten gedestabiliseerd raken en gaan lekken. Hierdoor zou het onder andere mogelijk zijn dat MBL uit de vaatjes lekt, vervolgens bij de epitheelcellen kan komen en de tubulus epitheelschade veroorzaakt. Naast het wegvangen van MBL zou een therapie gericht op het stabiel houden van de bloedvaatjes daarom ook succesvol kunnen zijn. Ten eerste omdat dan voorkomen zal worden dat het MBL uit de bloedvaatjes lekt en bij de tubulus epitheelcellen kan komen. Ten tweede wordt de vorming van oedeem voorkomen en ten derde zal de ophoping van ontstekingscellen die uit het bloed komen, ook minder zijn.

Conclusie

Het complementsysteem speelt een belangrijke en gunstige rol in het opruimen van ziekteverwekkers en dode cellen in ons lichaam. Echter, bij een niertransplantatie kan complementactivatie nadelig zijn en de I/R schade juist verergeren door het vrijkomen van C3a en C5a, en de vorming van het C5b-9 complex. Studies in muizen hebben een belangrijke rol aangetoond voor de alternatieve route van complementactivatie op (ischemische) tubulus epitheelcellen. In dit proefschrift

hebben we aangetoond dat bij ischemische menselijke tubulus epitheelcellen de klassieke route van complementactivatie betrokken is, wat laat zien dat er belangrijke verschillen zijn in het tubulusepitheel en complementsysteem tussen muis en mens. Daarnaast hebben we laten zien dat slechts enkele seconden na reperfusie er enige mate van complementactivatie in de circulatie optreedt, terwijl deze volledig afwezig is op de tubulus epitheelcellen. Ook hebben we aangetoond dat in de urine spontane complementactivatie kan optreden wanneer bloed in contact komt met urine. Dit proces is volledig onafhankelijk van de nier en maakt het lastig om betrouwbare complementmetingen te verrichten in de urine.

In het I/R model in de rat hebben we vervolgens laten zien dat complementactivatie pas optreedt nadat er al I/R schade is ontstaan. Complementactivatie na I/R is dus meer een secundair proces en ligt niet ten grondslag aan de initiële I/R schade in de nier. Complementactivatie op beschadigde en dode tubulus epitheelcellen treedt uiteindelijk wel op, maar op een relatief laat moment na reperfusie. De belangrijkste vinding van dit promotieonderzoek is dat echter vroeg na reperfusie, MBL uit het bloed in de nier terechtkomt en wordt blootgesteld aan de tubulus epitheelcellen. MBL heeft vervolgens een cytotoxische (celdodende) werking op deze cellen. De cytotoxische werking van MBL is echter volledig onafhankelijk van complementactivatie. Het remmen van C3 en C5 beschermde daarom ook niet tegen I/R schade, terwijl het wegvangen van MBL bijna volledige bescherming bood. Ook konden we laten zien dat de blootstelling van MBL en het dood gaan van de tubulus epitheelcellen voorafging aan de activatie van het complementsysteem. MBL is daarom een zeer interessant therapeutisch doelwit en het tijdelijk remmen of wegvangen van MBL tijdens een klinische niertransplantatie zou daarom een veelbelovende behandeling kunnen zijn om I/R schade na niertransplantatie te voorkomen.

LIST OF ABBREVIATIONS

ABTS	2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
AKI	acute kidney injury
ANG-1	angiopoietin-1
ANG-2	angiopoietin-2
AP	alternative pathway
ATF6	activating transcription factor 6
ATN	acute tubular necrosis
ATP	adenosine triphosphate
BDD	brain dead donor
BUN	blood urea nitrogen
CDD	cardiac dead donor
COMP	cartilage oligomeric matrix protein
CP	classical pathway
CRD	carbohydrate recognition domain
CRP	complement regulatory protein
CVF	cobra venom factor
DAB	3,3’-diaminobenzidine
DAMP	danger-associated molecular pattern
DC	dendritic cell
DGF	delayed graft function
DIG	digitonin
EC	endothelial cell
ER	endoplasmic reticulum
ERAD	ER-associated protein degradation
FITC	fluorescein isothiocyanate
GlcNAc	N-acetyl-D-glucosamine
GPR78	78-kDa glucose-regulated protein
HMGB1	High mobility group box 1
HPF	high power field
HRP	horseradish peroxidase
HSA	human serum albumin
HTK	histidine–tryptophan–ketoglutarate

HUVEC	human umbilical vein endothelial cell
I/R	ischemia/reperfusion
IM-PTEC	immortalized mouse proximal tubular epithelial cells
IRE-1	inositol-requiring enzyme-1
IRI	ischemia/reperfusion injury
KIM-1	kidney injury molecule-1
LD	living donor
LP	lectin pathay
mAb	monoclonal antibody
MAC	membrane attack complex
MASP	MBL-associated serine protease
MBL	mannan-binding lectin
MCP-1	macrophage chemoattractant protein-1
Mph	macrophage
NGAL	neutrophil gelatinase-associated lipocalin
NHS	normal human serum
NMS	normal mouse serum
PDGFRβ	platelet derived growth factor receptor β
PDI	protein disulfide isomerase
PERK	pancreatic ER kinase
PI	propidium iodide
PMN	neutrophil
PO2	partial pressure of oxygen
PRR	pattern recognition receptors
PTEC	proximal tubular epithelial cell
RECA-1	rat endothelial cell antigen-1
rhC1INH	recombinant human C1 inhibitor
ROS	reactive oxygen species
RPS-15	ribosomal protein S-15
SD	standard deviation
SEM	standard error of the mean
SNP	single nucleotide polymorphism
TLR	toll-like receptor
UPR	unfolded protein response
UW	University of Wisconsin

CURRICULUM VITAE

De schrijver van het proefschrift, Pieter van der Pol, werd geboren op 21 maart 1983 in Rotterdam. In 2001 behaalde hij het VWO diploma aan het Wartburg College te Rotterdam. In datzelfde jaar begon hij met de studie Life Science & Technology aan de Universiteit Leiden en Technische Universiteit Delft. Tijdens deze studie verrichtte hij een Bachelor onderzoeksstage op de afdeling Nierziekten van het LUMC te Leiden onder supervisie van Dr. A. Roos en Prof. Dr. M.R. Daha. Tijdens deze stage deed hij onderzoek naar de interactie tussen Mannan-binding lectin en humaan polymeer serum IgA in het kader van IgA nefropathie. De Master onderzoeksstage tijdens deze opleiding verrichtte hij op de afdeling Virologie van het Erasmus MC te Rotterdam onder supervisie van Dr. B. Martina en Prof. Dr. A.D.M.E Osterhaus. Tijdens deze stage werd onderzoek gedaan naar de immuunrespons tegen het West-Nijlvirus in C57BL/6 muizen. De studie Life Science & Technology werd afgesloten in 2006 met de Master "Functional Genomics". Aansluitend hierop begon hij in oktober 2006 aan een Nierstichting-gefinancierd promotieonderzoek getiteld "The role of the lectin pathway of complement in renal ischemia/reperfusion injury and transplant rejection" op de afdeling Nierziekten van het LUMC onder supervisie van Prof. Dr. M.R. Daha en na diens emeritaat onder supervisie van Prof. Dr. C. van Kooten. De resultaten van het promotieonderzoek zijn beschreven in dit proefschrift. Eind 2011 ontving hij een Kolff Postdoc Startup beurs van de Nierstichting getiteld "The role of ER-stress and autophagy in MBL-mediated tubular epithelial cell death following renal ischemia/reperfusion". Vanaf 2012 verricht hij op dit onderwerp postdoctoraal onderzoek in de onderzoeksgroep van Prof. Dr. C. van Kooten.

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