

Ischemia-reperfusion injury in kidney transplantation

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1. ABSTRACT

Ischemia-reperfusion injury to the kidney is a complex pathophysiological process that has importance during transplantation as it affects graft function and survival. It starts with the physiological changes associated with the death of the donor, including the direct effects of hypoxia and metabolic stress. The injury continues through the organ procurement and preservation procedures. Upon reperfusion, the organ is then further damaged by a reactive inflammatory process which had been primed during the earlier injuries. Clinically, the damage from microvascular dysfunction and cytotoxic agents contributed by the immunologic response results in impaired graft function or graft loss. Recent advances in understanding the specific pathways involved in this injury have helped identify novel therapies. Nevertheless, ischemia-reperfusion injury continues to be a daunting problem even as these treatment strategies are being evaluated for clinical use.

2. INTRODUCTION

In kidney transplantation, organ injury begins with the physiologic changes associated

with brain death or circulatory death and continues through implantation. After removal from the donor, the organ is maintained for a short period in nonphysiologic conditions before ultimately being reperfused at the time of transplant. During this entire time, a complex pathophysiological process results, beginning with the altered perfusion and ischemia associated with organ retrieval and preservation and then continuing with damage after reperfusion from the ensuing pro-inflammatory milieu. These complex processes are known as ischemia-reperfusion injury (IRI) and manifests clinically as delayed graft function (DGF) or primary non-function (PNF) after transplantation.

In this review, we will define DGF with its adverse consequences and describe various diagnostic methods. We will then review the mechanisms involved in the pathophysiology of IRI and potential treatments. This discussion will be organized into two parts: those that apply (1) to the donor and the ischemic injuries prior to implantation or (2) to the recipient and the reperfusion injuries

after implantation. Of course in the continuum of injuries, these delineations do not always neatly partition along a particular etiology or treatment strategy. Processes involved in IRI can begin with ischemia and extend well past reperfusion to effect chronic changes. Similarly, therapeutic interventions can often be applied before implantation and maintained afterwards. The review will focus chiefly around those factors around the immediate time of transplantation which may influence IRI specifically.

2.1. Delayed graft function

DGF describes general renal dysfunction after transplantation usually due to IRI, but may be the result of other insults such as acute rejection or drug nephrotoxicity. Classically, DGF is defined as the requirement for dialysis within 7 days of transplant (1) and is distinguished from PNF by eventual recovery of renal function. This definition has not been universally accepted, however, because the criteria for initiation of and timing of dialysis post transplantation varies significantly from center to center (2, 3), while other definitions of DGF use a variety of measures of kidney function such as oliguria (4) or rate of creatinine clearance (5). There is also a less severe form of impaired graft function referred to as slowed graft function (SGF), which is defined as serum creatinine greater than 3 mg/dL on postoperative day 5 of transplantation, without needing dialysis (6). Although more research is required, initial reports suggest both the SGF and DGF groups are at similar risk of poor graft outcomes (7). Grafts with immediate graft function lack characteristics of SGF and DGF.

The different definitions and criteria for defining DGF between centers likely explain the range of reported DGF incidence across studies which range from 8% to 50% (8). Other factors, such as an increasing pool of extended criteria donor (ECD) grafts may also play a role. For instance an early series of patients transplanted from 1985 to 1992 reported DGF rate of 14.1% (9), but a later series of patients from 1998 to 2004 reported a rate of 23% (10). In living related donors, the rate of DGF is lower, between 4 to 10% (8, 11).

DGF is associated with increased risk of allograft rejection, both acute and chronic, ultimately resulting in graft loss (12, 13). However, some investigators argue that this correlation may be a result of diagnostic bias where rejection is observed more frequently because more DGF patients are biopsied (14). A meta-analysis reported an increased

risk of 36% for acute rejection and 41% for graft loss in DGF versus non-DGF (15). Although this same review noted no change in overall patient survival with DGF, consistent with other reports (16), a recent analysis of patients from 1998 to 2004 showed initial DGF was associated with an increased risk of death with a functioning graft (10). DGF is associated with a longer hospital stay and being on dialysis, which carry an added economic cost of approximately \$5000 per transplant (17).

2.2. Diagnostic tools

While the clinical consequences of IRI manifesting as DGF are detected on the order of days after transplantation, early diagnosis within hours utilizing molecular diagnostic and other techniques can potentially open a window for more effective therapies (3). Postoperative biopsies remain the gold standard by which to evaluate the cause of DGF (18) especially to rule out acute rejection. DGF typically results in histological features consistent with acute tubular necrosis (ATN) (19). Many centers will biopsy a kidney that is not functioning at seven days to rule out other etiologies such as acute rejection.

Although a section on biomarkers is reported elsewhere in this series, it is worth pointing out several promising biomarkers for assessing renal function promptly. Neutrophil gelatinase-associated lipocalin (NGAL) is a protein expressed in epithelial cells whose role is unclear, but may be pivotal in regeneration of the proximal tubule after injury (20). In kidney injury, high levels of NGAL can be measured in the urine. IL-18 is a pro-inflammatory cytokine, likely produced during IRI (21). In one prospective study, both NGAL and IL-18 were able to predict DGF from measurements on post-operative day 1 (22) where serum creatinine alone was not predictive. Another biomarker, kidney injury molecule-1 or KIM-1, is a cell membrane glycoprotein which has been implicated in converting proximal tubular epithelial cells to a phagocytic phenotype. Its extracellular domain is shed during injury and can be detected in the urine, rising sharply in the first 24 hours (23) of IRI with better receiver-operator characteristics than blood urea nitrogen or serum creatinine in rat. In a transplant setting, staining of biopsies with KIM-1 correlated with tubular injury and subsequent renal dysfunction (24) even when the histology was inconclusive.

Beyond biomarkers, various imaging methods have been used to study DGF, showing decreased regional blood flow compared with

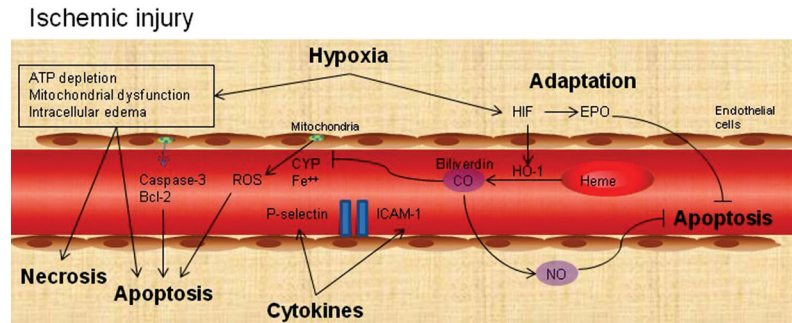


Figure 1. Schematic of processes in ischemic injury. *Hypoxia* leads to cellular processes which result in energy depletion, mitochondrial dysfunction, and intracellular edema. The end result is *apoptosis* or *necrosis* of the cell. At the same time, *adaptation* to hypoxia occurs via the HIF transcription factor and its downstream effectors, including HO-1. The endothelial cell is also primed by systemic *cytokines*.

immediately functioning grafts (25). Intravital microscopy in dogs undergoing ischemia showed an early decrease in blood flow within peritubular capillaries after reperfusion, which persisted (26). When adapted for human use (27) the images revealed a correlation between blood flow shortly after reperfusion and duration of ATN, and showed reduced blood flow impairment in living donors when compared to deceased donors. The use of Tc-99m mercaptoacetyl triglycine renal scan has been described (28) to predict DGF, however its use has not been widely adopted, likely secondary to expense and low specificity (3). Thermodiffusion measurements have been used to quantify perfusion in the renal cortex by measuring the rate of convective heat loss (29). Initial data in human experiments showed that measurements at 60 min after graft reperfusion correlated with subsequent renal function; however, an obstacle to its adoption is that the device must be inserted directly into the parenchyma.

3. ISCHEMIC INJURY AND MANAGEMENT OF DONOR ORGAN

The ischemic insult to the organ begins with the circumstances surrounding the death of the donor. Transient ischemia may occur with the initial event that caused the irreversible brain damage prior to procurement and there are also well-described physiologic changes associated with brain death that can further injure the organs. The term warm ischemia time (WIT) typically refers to the time period from removal of the organ from cold storage until it is reperfused at the time of transplantation. In donors after circulatory determination of death (DCDD) WIT refers to the time from declaration of death until cold perfusion of the organs. Cold ischemia time

(CIT) refers to the period of time from perfusion of the organs with cold preservation fluid at the time of retrieval until the organ is reperfused. Prolonged WIT or CIT can affect the underlying processes ultimately leading to IRI. Figure 1 summarizes the processes involved in ischemic injury which will be described in further detail.

3.1. Mechanism of ischemic injury

Ischemia refers to the deprivation of oxygen and nutrients to tissue from restricted blood circulation. Ischemic injury leads to a cascade of cellular processes ultimately culminating in cellular death. As aerobic metabolism halts, adenosine triphosphate (ATP) stores are diminished and ATP synthase becomes dysfunctional. There is an accumulation of byproducts of anaerobic metabolism resulting in acidosis and increased intracellular hyperosmolarity, which is further compounded by influx of extracellular sodium and calcium ions as energy-dependent transporters fail. Influx of water from the resulting osmotic imbalance results in cellular edema which then further disrupts cellular function as well as structural integrity of organelles such as the endoplasmic reticulum and mitochondria. Increasing endoplasmic reticulum stress is a result of accumulating misfolded proteins from glucose deprivation and redox state imbalance triggering an unfolded protein response that leads to apoptotic pathways (30). Mitochondrial damage from calcium overload and reactive oxygen species (ROS) causes permeability transition pores to open (31). Leakage of cytochrome c from the inner matrix can activate the caspase pathway in apoptosis. When the mitochondrial membrane potential required for oxidative phosphorylation is lost and ATP is depleted, the process is irreversible and cellular necrosis results.

Although cold preservation was developed to reduce anaerobic metabolism and the sequelae described above, these processes are not completely halted. Studies involving cold storage of human proximal tubular epithelial cells showed that cell death was predominantly from necrosis, with signs of mitochondrial swelling and membrane disruption (32). Upon rewarming and reperfusion, there is a switch to apoptosis of the epithelial cells (33). Additional evidence suggests that with as little as 2 hours of CIT, there is an increase in mitochondrial permeability transition pores, with translocation of cytochrome C and subsequent activation of the Bcl-2 and caspase-3 pathways (34). As the mitochondrial respiratory complexes begin to fail during ischemia, ROS are formed. Though ROS are a well known cause of reperfusion injury, as discussed later, there is increasing evidence that increased oxidative stress can also occur during cold storage, with one possible cause being the deactivation of superoxide dismutase (SOD) (35). A second mechanism is thought to be release of free-iron from cytochrome P450 in microsomes (36). Free iron is noted to be a source of ROS, and initial studies suggest that addition of the iron chelator, deferoxamine, to preservation fluids improved GFR after transplantation in a dose dependent mechanism while reducing lipid peroxidation byproducts (37).

3.2. Hypoxia adaptation

Despite the mechanisms of ischemic injury described above, the organ has several notable protective strategies to handle hypoxic stress. One cellular response to hypoxia is mediated through the hypoxia inducible factor (HIF), a transcription factor which subsequently activates multiple targets including vascular endothelial growth factor (VEGF), erythropoietin (EPO) and heme-oxygenase-1 (HO-1) (38). Blocking HIF inactivation by inhibiting HIF hydroxylases has been shown to protect kidneys against IRI in animal models (39). Interestingly, in this study, there also appears to be a secondary activation of HIF during reperfusion, which is thought to be triggered by the pro-survival Akt/m-TOR pathway rather than hypoxia.

Because of the role of HIF in protecting against hypoxia, the downstream targets of HIF have been actively studied as therapeutic targets in IRI. EPO is best known for its role in erythropoiesis, but pre-clinical studies implicated a possible protective role in ischemia (40). The EPO receptor activates multiple pro-survival and anti-apoptotic pathways, but the exact protective mechanism is not well

elucidated (41). Nevertheless, randomized clinical trials using recombinant human EPO have not shown a reduced rate of DGF (42, 43). A possible explanation is that the protective mechanisms are mediated via a heteromeric association of the EPO receptor and a beta-common receptor (44), and that the dose required to activate this heteromeric complex is actually much higher than was used in the trials. High dose EPO administration has potential complications related to vascular thrombosis (41), and thus novel analogues to EPO that are more specific for this heteromeric complex are currently being explored (44).

Another target of HIF is HO-1, a protein involved in heat-shock and stress response which is best known for its role in breaking down heme into biliverdin, Fe^{++} , and carbon monoxide, but that also has been shown to have anti-inflammatory, anti-apoptotic and anti-oxidant properties (45). Early work with HO-1 in IRI suggested that hyperthermic preconditioning of rodents upregulated HO-1 resulting in protection from IRI (46). Later work showed that direct induction of HO-1 by cobalt protoporphyrin prior to organ harvest resulted in significant improvement in graft function as well as reduction in apoptotic markers and IRI seen on histology (47). Another method of inducing HO-1 during cold storage is via the dopaminergic agonist fenoldopam (48). There is increasing evidence that some of the effects of HO-1 are directly the results of the byproducts of heme catabolism, in particular carbon monoxide (CO) and biliverdin (45). While the role of biliverdin is largely as an antioxidant, CO has received significant attention for being the effector molecule in the downstream effects of HO-1 (49).

Although at large doses of CO there is toxic competitive binding with heme to produce hypoxia, at lower doses, CO has been shown to attenuate renal IRI via a number of intriguing pathways that are anti-inflammatory, anti-apoptotic, and vasodilatory (50). One proposed mechanism is that CO interacts with various enzymes that have heme moieties because of its high iron affinity. One particular enzyme is cytochrome P450, which as mentioned earlier is a potential source of ROS when degraded. Evidence shows that CO stabilizes CYP450, thus reducing its degradation and release of heme (51). Another target of CO with a heme component is serum guanylyl cyclase (sGC), a known receptor for nitric oxide (NO), which results in vasodilation (52). In pre-clinical trials with pigs, CO given during cold storage significantly reduced

mRNA levels for proinflammatory cytokines as well as lipid peroxidation, resulting in improved survival and graft function with kidney transplantations (53). Similar results were reported with the use of CO releasing molecules (CORMs), with improved renal perfusion, glomerular filtration rate, and mitochondrial respiration after transplantation (54). Although there is a theoretical benefit of delivering CO to the organ while cold stored there are studies showing that CO treatment of the recipient is also effective. In a rat model, CO delivery to the recipient perioperatively showed improved graft function versus control animals (55). The study reported increased mRNA levels of HIF and VEGF as well as higher levels of NO production, suggesting that CO promoted repair pathways and vasodilation to protect the endothelial cells. In a pig model of warm ischemia, CO treatment of the recipient animal for 1 hour showed improved outcomes for untreated donor grafts with WIT of 60 minutes and 24 hours of CIT (56). Data from this study showed CO administration reduced immunogenicity, decreased apoptosis, and promoted proliferation of tubular epithelial cells resulting in earlier return of graft function.

Since hypoxia triggers protective mechanisms described above, investigators have used ischemic preconditioning (IP) as a strategy for augmenting the hypoxic adaptive responses prior to transplantation (57). IP results in an early effect, usually minutes or hours after conditioning, as well as a late effect that appears on the order of days later (58). One early report used a 15 minute warm ischemia time with direct clamping of the renal pedicle followed by 10 minutes of reperfusion prior to transplantation in rats (59). The results suggested improvements with preconditioning post transplant, possibly mediated by upregulation of NO locally. However, increasing the time of reperfusion abolished the protective mechanism. Another study in rats used repetitive hypoxic preconditioning in a non-transplant warm ischemia model that showed upregulation of HIF, decreased oxidative stress, and reduced apoptotic markers (60). Later studies with large animals, however, did not show protection from IRI (57). Little has been reported in human trials, with a significant barrier arising from an absent consensus protocol on how IP should be implemented (58).

3.3. Donor physiology and management

Kidneys can be utilized from living donors (LD) or deceased donors (DD). DD organs are further classified as donation after brain death (DBD) or donation after circulatory determination of death

(DCDD). DBD and DCDD can then be further divided into standard criteria donors (SCD) or expanded criteria donors (ECD). While it is well documented that organs from LD have better graft survival than DD (61, 62), specific features of organs from the other donor classifications contribute to their poorer outcomes related to IRI.

In DBD, the processes leading to brain death often result in a catecholamine storm which produces hemodynamic instability, vasoconstriction and organ hypoperfusion. Neuronal death results in accumulation of pro-inflammatory cytokines. As the blood-brain barrier degenerates, these cytokines are released into systemic circulation, thus priming the local inflammatory state of peripheral organs (63), resulting an increase in adhesion molecules on the endothelium (64), increased cytokines (65), and infiltration of immune cells into DD organs compared to LD organs (66). The role of increased graft immunogenicity will be discussed below, but as one example of its therapeutic potential, treating donor rats after brain death with an antagonist to the adhesion molecule, P-selectin, significantly reduced subsequent renal injury after transplantation (67). Because of hemodynamic instability in DBD, donors are often managed aggressively in an intensive care setting. However, these treatments may themselves have adverse effects on the kidneys. One retrospective study of SCD kidneys showed in a multivariate analysis, epinephrine use during this resuscitative period correlated with prolonged DGF, whereas judicious volume expansion and longer ICU stays decreased DGF (68). In a randomized control trial, dopamine use has been demonstrated to improve organ yield (69), with likely mechanisms related to reduction of oxidative stress and endothelial cell protection (70), as opposed to stabilization of hemodynamics.

DCDD, previously known as donors after cardiac death or non-heart beating donors, differ from DBD in that the kidneys undergo a period of warm ischemia time after extubation, through cardiac arrest, and before cold perfusion. Higher rates of DGF for DCDD organs than DBD organs have been noted. Studies of DCDD showed that CIT less than 12 hours and donor age less than 50-60 years are associated with decreased DGF (71, 72). However, despite the rate of DGF, outcomes in patient and graft survival for up to 10 years are not different between DCDD and DBD (73, 74), with one series even noting a 23% decreased rate of graft loss in DCDD (71). It is not well understood how DCDD

organs appear to be protected from long-term effects of DGF when compared to DBD organs. A likely difference may be how brain death activates organs immunologically in ways not experienced by DCDD organs (75). Despite the increased WIT associated with DCDD, the resulting injuries from warm ischemia leading to DGF are perhaps reversible. Whether there is a threshold amount of WIT before graft quality deteriorates is unknown (76), in part because it unclear if WIT itself is the best marker for the injury.

ECD refers to donors that meet specific criteria derived in a retrospective analysis of their characteristics (77) that were found to result in 70% higher risk of graft failure versus SCD and include: 1) all donors greater than 60 years of age; or 2) donors greater than 50 years of age with two of three risk factors (*i.e.* hypertension, renal insufficiency with serum creatinine greater than 1.5. mg/dL or history of cerebrovascular accident as the cause of death). Although the increased risk of graft failure is inherent in the definition of ECD, there may be greater variability in the quality of organs that meet these criteria (78). Pre-transplant evaluation may be helpful in predicting the outcome of an organ using markers such as glomerular filtration rate estimation or donor kidney biopsy and assessing the presence of arteriosclerosis and ECD kidneys tend to have increased rates of DGF with prolonged CIT but longer CIT does not appear to impact long-term graft survival (80).

3.4. Preservation strategies

During static cold storage of the organ, preservation fluids have been designed to minimize the problems of electrolyte imbalance, oxidative stress, and cellular edema associated with cold ischemic injury. Although a review of human trials regarding commonly used preservation fluids is reported elsewhere (81, 82), results continue to show UW and HTK solutions to be superior (83). Whether UW is superior to HTK remains an area of controversy, with some evidence of reduced graft survival in HTK (84) and other evidence suggesting no differences between the two (85). Numerous therapeutic agents have been investigated as additives to preservation fluids. These studies have been extensively reviewed elsewhere (81, 86) and target many of the factors described above, including anti-apoptotic agents, antioxidants, and HIF modulators. One strategy to mention in particular is the use of anti-thrombin, which attempts to minimize microthrombosis during storage which can result in “no-reflow” after reperfusion (87). In a pig

kidney warm ischemia model, treatment of the graft during storage with Melagatran resulted in reduced rates of PNF, with further studies on endothelial cells showing reduced levels of the proinflammatory markers thrombospondin and P-selectin as well as oxidative stress (88).

One area of continued investigation is the use of cold machine perfusion (MP) to preserve organs instead of cold storage. One meta-review showed 20% reduction in relative risk of DGF in MP kidneys vs. cold storage, but no overall benefit in 1 year graft survival (89). A subsequent multicenter European randomized control trial with paired kidneys also showed a significant reduction in DGF as well as improved graft survival at 1 year (90). In DCDD allografts randomized control studies have supported (91) and rejected this hypothesis (92). Meta-analysis in the latter study noted that although the DGF rates in the MP group were actually similar between the two DCDD trials, the rate of DGF in the cold storage group was slightly higher in the former study, possibly from longer WIT. Continuing investigation will be needed to sort out the methodological differences between the trials to reach a firm conclusion of using MP in DCDD. In ECD allografts, an extension of the European randomized control trial showed improvements in DGF rates, PNF rates, as well as 1 year graft survival (93). This result is in contrast to an earlier study, which also described improvements in DGF rates, but showed no overall graft survival up to 3 years (94). One possible difference is that the CIT in the latter study was 20 hours vs. 13 hours in the European study. While results from these studies tend to show reduction in DGF with MP, predicting which organs may benefit from DGF with MP remains an area of research, especially with respect to the mechanisms of IRI. MP itself may allow further evaluation of the graft quality biochemically and functionally prior to transplantation (81). There is also a recent report of using *ex vivo* normothermic perfusion (95) in ECD kidneys, which is another step towards maintaining physiologic conditions during preservation. Although the results showed lower DGF compared to cold storage, 1 year graft and patient survival were not significantly different.

4. REPERFUSION INJURY AND MANAGEMENT OF RECIPIENT ORGAN

Reperfusion injury represents a derangement of reparative and inflammatory pathways reacting to the ischemic insults described

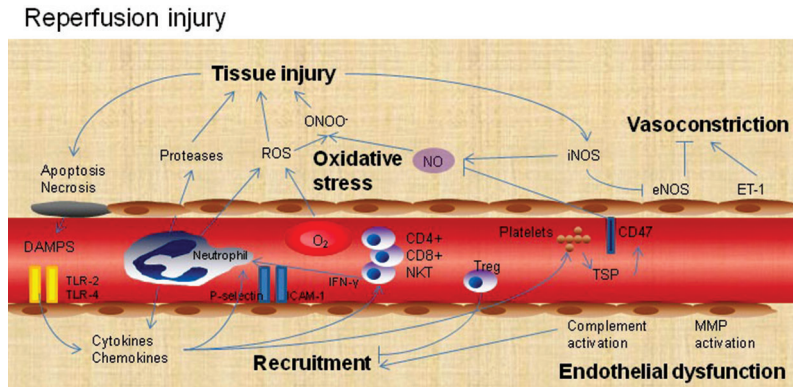


Figure 2. Schematic of processes in reperfusion injury. Reoxygenation leads to a sudden increase in oxidative stress. Tissue injury activates the endothelium and the innate immune system which in turn leads to recruitment of immune cells via production of cytokines and chemokines as well as the help of adhesion molecules. Neutrophils add to the oxidative stress and secrete cytotoxic agents, all of which contribute to tissue injury. At the same time, endothelial injury results in suppression of eNOS which leads to an imbalance of vasoactive substances favoring vasoconstriction. Endothelial injury also increases the permeability of the microvasculature, producing hemoconcentration, microthrombosis and obstruction.

above. Upon restoration of oxygen with reperfusion, there is a sudden burst of ROS production, which causes direct injury to cellular membranes and DNA as well as an inflammatory response. In addition, as cells die from the injury, normally intracellular products are released and activate pattern-recognition receptors which trigger the innate immune system, activate the complement system and increase cytokine and chemokine production. There is then recruitment of immune cells which carry a directly cytotoxic phenotype. At the same time, the endothelial cells of the microvasculature upregulate adhesion molecules, produce an excess of vasoconstrictive substances, adopt a pro-thrombotic state, and have increased permeability. The net result of this sterile inflammatory process is that the already injured tissue suffers from another hit of cytotoxic processes as well as reduced perfusion from microvascular collapse. Figure 2 summarizes the major pathways which will be reviewed.

4.1. Reactive oxygen and nitrogen species

During ischemia, adenosine is deaminated to hypoxanthine which is then metabolized by xanthine oxidase (XO) upon reintroduction of oxygen during reperfusion. XO is a major source of ROS production during reperfusion (96), and an excess amount overwhelms the normal antioxidant mechanisms. ROS directly cause lipid peroxidation, whose byproducts are noxious and damage membrane lipids to increase the permeability of cells and organelles (97). ROS thus have direct effects on

mitochondria, which when damaged, as discussed earlier, leads to activation of apoptosis (98). Although a more comprehensive review of antioxidants in treating IRI exists in the literature, their utility remains to be demonstrated in a clinical trial (99).

Nitric oxide (NO) is a small signaling molecule that causes vasodilation via the soluble guanylate cyclase and is implicated in antioxidant and anti-apoptotic pathways (100). It is normally produced via various isoforms of nitric oxide synthase (NOS), including the endothelial NOS (eNOS) and inducible NOS (iNOS). Evidence suggests that eNOS inhibition plays an important role in the resulting endothelial dysfunction (98). One recently discovered regulator of NO is via the thrombospondin (TSP) and CD47 (integrin associated protein) axis (101). In its normal role during injury, TSP is secreted by platelets to produce vasoconstriction via CD47 mediated inhibition of soluble guanylate cyclase and eNOS via reduced calcium influx (102). CD47 null mice and animals treated with antibodies to CD47 show protection from IRI (103). As opposed to eNOS, iNOS is induced during IRI (100) and inhibition of iNOS confers cytoprotection on proximal tubular epithelial cells (104). One consequence of simultaneous increased oxidative and nitrosative stress is the production of the peroxynitrite anion (ONOO⁻), which directly causes lipid peroxidation, DNA damage, and nitrotyrosine modification of proteins (98). DNA damage in particular causes further damage by depleting cellular ATP levels via poly (ADP-ribose) polymerase-1 (PARP-1)

dependent activation of DNA repair enzymes and inhibition of glycolysis (105). Scavenging or inhibition of peroxynitrite anion increases renal function after warm ischemia in a rodent model (97).

4.2. Innate immune response

Many of the systems that respond to microbial invasion are activated in reperfusion injury. Of the innate system, pattern-recognition molecules such as the Toll-like receptors (TLRs) respond to “damage-associated molecular pattern” (DAMP) ligands released from cells during injury, such as heat-shock proteins, DNA, the high-mobility group box 1 (HMGB1) protein, or ATP (106, 107). TLR acts via the myeloid differentiation-primary response adapter protein (MyD88) to activate the NF-kappa B transcription factor, and TLR activates interferon pathways independently of MyD88 (108). Ultimately TLR increases expression of various cytokines and chemokines. Upregulation of TLRs, in particular TLR2 and TLR4 (109), has been documented in kidney IRI. In a rodent model TLR4 expression and ligands such as HMGB1 were upregulated following IRI, with increased levels of neutrophil and macrophage infiltration as well as expression of TNF-alpha, IL-1 and IL-6 (110). TLR4 knockout strains had significantly reduced levels of cytokines and cellular infiltration along with decreased apoptosis of tubular epithelial cells and improved renal function after injury. In a human study, TLR4 levels were higher in grafts from DBD versus LD; however, grafts from donors with loss-of-function mutations in TLR4 had increased levels of not only TNF-alpha and MCP-1, but also HO-1 expression (111).

Release of cytokines and chemokines by local immune and endothelial cells then begin recruitment of leukocytes, particularly neutrophils in the first hour after reperfusion (112). Neutrophils produce additional oxidative stress via NADPH oxidase (8), secrete elastases which are directly cytotoxic, and further the recruitment of other immune cells, including monocytes, followed by T-cells. A more general review of cytokines and chemokines in IRI and transplantation models (113) shows that blocking these molecules decrease neutrophil infiltration and produce decreased tissue injury. The complexity of their interactions with the target cells, however, means that no one blockade alone will likely be effective (113). As mentioned, earlier, the production of these chemoattractants is also dependent on the conditions of the donor graft, with DBD showing higher levels than LD.

The complement cascade is another component of the innate system that is reportedly activated during IRI (114), although its role remains unclear. The C3 component, for instance, is induced upon brain death and remains elevated during reperfusion, with higher levels correlating with decreased renal function in human studies (115). In mouse models, complement causes injury through the membrane attack complex (MAC), which is the common endpoint of three different complement activation pathways (116). Nevertheless, in clinical studies, only transient levels of soluble C5b-C9 components in MAC were detected 30 minutes after reperfusing DBD grafts; and no local deposition in the renal vasculature or tubules was identified (117). The actual pathway leading to complement activation remains an area of controversy (114). Recently, mannose-binding lectin, which activates one complement pathway, has been implicated in IRI albeit through an apparently complement-independent process involving direct cytotoxicity to the tubular epithelial cells (118). The role of complements in IRI thus needs to be further elucidated, as there appears to be conflicting results between the various models and human data.

4.3. Adaptive immune response

The earliest evidence of T-cell involvement in IRI comes from experiments with CD4 and CD8 double knockout mice (119), where animals with the deficient T-cells have improved renal function at 48 hrs after reperfusion when compared to wild type. In a more recent experiment, pretreating rats with anti-thymocyte globulin to deplete T-cells 2 hours prior to kidney transplantation improved post-transplant renal function (120) without decreasing granulocyte influx. Despite this evidence, the actual role of CD4+/CD8+ T-cells in IRI is not clear, since T-cell activation is classically associated with antigen-presentation (121), which is a late process (122). One possible mechanism is that T-cell adhesion obstructs the capillaries in the renal microvasculature (120). Other T-cell subtypes are also increasingly being studied for their function in IRI. Natural killer T-cells (NKT) have been recently shown to be activated in IRI to recruit neutrophils via interferon- γ , and depletion of NKTs improved renal function after ischemia via reduced neutrophil influx (122). Another subtype, the regulatory T-cell, produces anti-inflammatory cytokines and has an immunosuppressive phenotype. Regulatory T-cells are now recognized to be protective in inflammatory processes such as IRI, and depletion of these cells worsens IRI in rodent models (123). Understanding

the pathways that lead to resolution of inflammatory processes, including those involving regulatory T-cells, may lead to further therapeutic targets in IRI.

4.4. Endothelial dysfunction

As discussed above, a key process in IRI beginning even before organ procurement is the upregulation of adhesion molecules on the endothelial surface, which subsequently allows neutrophils and other leukocytes to accumulate in the area of ischemic injury. Indeed, increased levels of adhesion molecules are correlated with DGF, with more expression in DBD versus LD grafts (124). Initial experiments with intracellular adhesion molecule-1 (ICAM-1) knockouts showed reduce neutrophil infiltration and improved renal function (125). Similar results were reported with the use of soluble P-selectin ligands (126). In human clinical trial, a P-selectin antagonist (YSPSL) was shown to reduce inflammatory cytokines (127); however initial results did not show improvements in DGF rate, although the study was not powered for efficacy (128). Similarly, a clinical trial of an anti-ICAM-1 antibody failed to improve DGF rates (129). When coupled with the increased congestion by leukocyte adhesion, release of local vasoconstrictors during IRI leads to a “no reflow” phenomenon after reperfusion. As noted above, NO, is one of the most studied local vasodilators. Antagonizing NO is endothelin (ET), which acts via ET receptors to induce vasoconstriction. ET receptor blockade in a rodent model attenuated IRI; however, its role in clinical use remains to be explored (130). As the processes of IRI continue to injure the endothelial cell layer, there is increased permeability in the microvasculature, which contributes to hemoconcentration and further worsens the vascular congestion (131). One mechanism which leads to the increased permeability is upregulation of matrix metalloproteinases (MMPs). In rat experiments, minocycline inhibits MMPs to reduce leakage of fluorescent dextrans in intravital microscopy (132) and has anti-inflammatory and anti-apoptotic properties that result in improved renal function after ischemia (133).

5. DESIGNING CLINICAL TRIALS

Despite the increasing understanding in IRI pathophysiology, translating potential therapeutic strategies into clinical application has been a slow process, and evidence based on equivalent rates of DGF over decades suggests that there has been little effective change. To conclude this review, some

of the challenges in going from bench to bedside will be highlighted specifically with respect to IRI and transplant. Although a more general review of trial design and potential pitfalls can be found elsewhere (134), it is worth mentioning that typical pitfalls of clinical trial design, such as creating well-matched populations, having a sufficiently large study size, and defining explicit endpoints, naturally apply to trials in kidney transplantation as well (82). As the review of various trial results described earlier has shown, disagreements while studying similar problems tend to stem from inconsistent practices (e.g. using inconsistent definitions of DGF or maintaining different amounts of CIT) that pervade the field of transplantation.

One particular problem specific to IRI, as mentioned in the introduction, is defining endpoints with respect to the nonspecific clinical manifestations of IRI. Conventional markers of renal injury may be influenced by other factors like the pre-transplant function of the donor organ, use of post-transplant nephrotoxic agents, type of immunosuppression, or rejection. These other factors may confound the use of markers such as DGF or graft survival as a way of establishing the efficacy of any particular strategy in managing IRI. Because IRI is an acute phenomenon relative to the expected life of the graft, the need for more reliable and timely diagnostic markers of renal injury is paramount. Conventional markers of renal injury and other clinical endpoints such as graft loss may complicate the analysis and utility of the trial results if they do not accurately reflect the timing and pathophysiology of IRI (135).

Another problem is the need for multicenter trials. An experimental treatment may be administered at various stages around the time of transplant, potentially even to both the donor and the recipient. Before transplantation, however, the organs may be transported from one facility to another, and thus both facilities would have to be participants in the trial. One particular way this problem arises is in the use of paired kidney studies, which are particularly attractive, because one organ may be randomized to a particular treatment arm prior to being transplanted. Restricting the study to patients in a single center may hinder recruitment. Furthermore, a multicenter trial may be able to account for effects of variable institutional practices, so long as there is enough enrollment to measure the effects. Designing multicenter trials in transplant research, however, is a slow and potentially costly process. One obstacle in particular is the use of

multiple institutional review boards (IRBs). Protocols approved by IRBs of the sponsoring institution may not be approved in other participating institutions. A concern of a local IRB may be shouldering responsibility for potential lapses of the sponsor in the trial protocol. Despite the extra scrutiny, individual IRBs do not routinely communicate their concerns to each other. Multi-site IRB review would likely worsen variability and bias to the protocol as each IRB modifies the protocol to suit their local concerns, problems that paradoxically counter the advantage of multicenter trials in controlling variable practices (136). One solution to this problem would be to create a central IRB that would oversee such multicenter protocols. This model is already in place with certain VA studies and is adopted by the National Cancer Institute (137).

Once the proposed treatment has been applied, however, the patients need to be monitored serially. Because the time window for intervening in IRI is narrow yet the relevant clinical endpoints, such as chronic allograft nephropathy or graft loss, are long-term, designing a clinical trial with such large discrepancies in time scale poses a problem. In order to follow a sufficient number of patients to power analysis, sophisticated databases will be required to maintain long-term records of patients as they migrate to different clinics. A centralized system will keep, for instance, details of the trial participation, such as the particular treatment arm that was applied, and other key events related to the clinical endpoints of interest. These problems may be addressed as a national discourse regarding the benefits of electronic health records helps to motivate development of networked information systems.

6. SUMMARY

Ischemia-reperfusion injury (IRI) is a complex problem affecting all kidney transplants through its impact on the organ's immediate and long-term function. Continuing research in IRI treatment strategies is motivated in large part by the increasing pressure to use marginal grafts which are more susceptible to the injury processes described in this review. There is ongoing progress in elucidating the mechanisms of IRI, particularly in the role of the graft's own adaptive response to hypoxia during ischemia and in detailing the interaction between the innate and adaptive immune system in causing reperfusion injury. While some of the proposed treatment strategies have shown promise in their efficacy, many still require large trials to demonstrate

their role in clinical use. Given the multiple points of initiating injury during transplantation, we predict that ischemia-reperfusion injury will ultimately be addressed by the serial application of multiple treatment strategies.

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