Ischaemia-reperfusion injury: a major protagonist in kidney transplantation

Claudio Ponticelli
Division of Nephrology, Scientific Institute, Milano, Italy
Correspondence and offprint requests to: C. Ponticelli; E-mail: ponticelli.claudio@gmail.com; claudio.ponticelli@fastwebnet.it

ABSTRACT

Ischaemia-reperfusion injury (IRI) is a frequent event in kidney transplantation, particularly when the kidney comes from a deceased donor. The brain death is usually associated with generalized ischaemia due to a hyperactivity of the sympathetic system. In spite of this, most donors have profound hypotension and require administration of vasoconstrictor agents. Warm ischaemia after kidney vessels clamping and the cold ischaemia after refrigeration also reduce oxygen and nutrients supply to tissues. The reperfusion further aggravates the state of oxidation and inflammation created by ischaemia. IRI first attacks endothelial cells and tubular epithelial cells. The lesions may be so severe that they lead to acute kidney injury (AKI) and delayed graft function (DGF), which can impair the graft survival. The unfavourable impact of DGF is worse when DGF is associated with acute rejection. Another consequence of IRI is the activation of the innate immunity. Danger signals released by dying cells alarm Toll-like receptors that, through adapter molecules and a chain of kinases, transmit the signal to transcription factors which encode the genes regulating inflammatory cells and mediators. In the inflammatory environment, dendritic cells (DCs) intercept the antigen, migrate to lymph nodes and present the antigen to immunocompetent cells, so activating the adaptive immunity and favouring rejection. Attempts to prevent IRI include optimal management of donor and recipient. Calcium-channel blockers, L-arginine and N-acetylcysteine could obtain a small reduction in the incidence of post-transplant DGF. Fenoldopam, Atrial Natriuretic Peptide, Brain Natriuretic Peptide and Dopamine proved to be helpful in reducing the risk of AKI in experimental models, but there is no controlled evidence that these agents may be of benefit in preventing DGF in kidney transplant recipients. Other antioxidants have been successfully used in experimental models of AKI but only a few studies of poor quality have been made in clinical transplantation with a few of these agents and we still lack of unambiguous demonstration that pre-treatment with these antioxidants can attenuate the impact of IRI in kidney transplantation. Interference with the signals leading to activation of innate immunity, inactivation of complement or manipulation of DCs is a promising therapeutic option for the near future. Keywords: acute kidney injury, delayed graft function, dendritic cells, innate immunity, ischaemia reperfusion

INTRODUCTION

Among the numerous variables that can influence the outcome of the transplanted kidney, an emerging risk factor is represented by ischaemia-reperfusion injury (IRI). Although IRI may also occur in kidney transplanted from living donors, it is more frequent and severe in kidneys of deceased donors. A number of factors may contribute to the pathogenesis of IRI. Brain death is associated with generalized ischaemia due to a hyperactivity of the sympathetic system aimed to maintain cerebral perfusion pressure. In spite of this, most donors have profound hypotension and require administration of vasoconstrictor agents that contribute to kidney hypoperfusion. Warm ischaemia after kidney vessels clamping and the cold ischaemia after refrigeration also reduce oxygen and nutrients supply to tissues. Manipulation of the kidney during removal may also produce mechanical injury. Moreover, brain injury and intracranial pressure may release massive amounts of cytokines and growth factors that can aggravate renal ischaemia [1]. Another consequence of brain death is local up-regulation and activation of complement that can aggravate the injury in the donor kidney [2]. Reperfusion of the ischaemic kidney further worsens the state of oxidation and inflammation.

In this paper, two main consequences of IRI that may influence the course of the transplanted kidney will be discussed, delayed graft function (DGF) and activation of innate immunity.
Endothelial cells and tubular epithelial cells are the first cells to suffer from the reduced oxygen supply. Anaerobic glycolysis and ATP degradation form superoxide radicals and an acidic milieu, which eventually result in phospholipolysis and cell membrane injury. Excess adenosine nucleotides activate the AMP kinase which limits the cell’s metabolic rate. Without ATP, Na/K ATPase exchangers cannot function leading to intracellular K+ and extracellular Na+ retention [3]. In this environment, the restoration of circulation results in inflammation and production of reactive oxygen species (ROS). White blood cells, carried by the returning blood, release a host of cytokines, chemoattractants, pro-coagulant factors and free radicals in response to tissue damage. Polymorphonuclear cells may bind to the endothelium and obstruct capillaries, so worsening ischaemia. Moreover, the restored blood flow reintroduces oxygen that damages the cell membrane, with further release of free radicals. During the early phase of ATP depletion, manganese superoxide dismutase (SOD), a major mitochondrial antioxidant that eliminates superoxide, is inactivated [4]. Thus, the oxidants mediate mitochondrial damage and activation of the receptors of apoptotic pathway, with consequent death of tubular epithelial cells [5]. These changes, which are usually proportional to the duration of ischaemic period, may eventually result in DGF (Figure 1).

Figure 1: Ischaemia reduces the oxygen supply to renal cells. ATP degrades forming superoxide. Excess adenosine nucleotides activate the AMP kinase which limits the cell’s metabolic rate. Without ATP, Na/K ATPase exchangers cannot function leading to intracellular K+ and extracellular Na+ retention. Oxidation and the acidic milieu result in phospholipolysis, altered cell membrane permeability and cell swelling. Endothelial and tubular epithelial cells are particularly vulnerable to ischaemia. After restoration of circulation, these lesions are worsened by the reintroduction of leucocytes (WBC) and oxygen. Leucocytes release cytokines, pro-coagulant factors and free radicals in response to tissue damage. Their binding to the endothelium causes capillary obstruction and further increases ischaemia. On the other hand, the supply of oxygen releases host of free radicals and damages the cell membranes, eventually leading to cell death. ROS, reactive oxygen species.

There is not a universally accepted definition of DGF. The traditional definition rests on dialysis requirement during the first post-operative week [6]. However, such a definition has been challenged, since the indications to dialysis are variable. Other classifications are based on creatinine reduction ratio and 24-h creatinine excretion from post-transplant Days 1–2 [7] or on the failure of the serum creatinine to decrease by at least 10% daily on three successive days during the first week post-transplantation [8]. Whatever the definition, DGF not only may require dialysis, but may prolong hospitalization, increase the complexity of the therapeutic approach and facilitate infections. Furthermore, in patients with DGF acute rejection or other injuries to the graft may remain undiagnosed, and a delay in detecting these complications may lead to irreversible parenchymal lesions.

A review of the United Network for Organ Sharing data found that DGF reduced the 5-year graft survival rate by 10–15% and shortened the half-life by about 2 years [9]. Some single-centre studies reported no impact of DGF on long-term graft survival in the absence of acute rejection [10, 11], but other investigators found that a DGF is associated with a poor graft outcome independently of rejection [12, 13]. Controversy also exists about the impact of slow graft function (SGF), defined by a slow improvement of serum creatinine in the first post-transplant week. Some investigators reported that SGF did not affect long-term graft survival, although it was associated with an increased incidence of acute rejection [14], while other authors reported that SGF had deleterious consequences on long-term graft survival, in recipients of kidneys from expanded-criteria donors [15].

The severity of acute kidney injury (AKI) can influence the outcome of the graft. After a mild AKI, the kidney has the ability to repair itself. However, when the injury is more severe or in the presence of kidney abnormalities, the repair process can lead to fibrosis, which can facilitate progression to chronic kidney disease [16]. Epithelial-to-mesenchymal transition (EMT) of injured epithelial cells plays a major role in the development of interstitial fibrosis. In this process, tubular epithelial cells progressively lose their epithelial characteristics and acquire features of mesenchymal cells. Hypoxia and several signal pathways are involved in the process of EMT. Of the many factors that regulate EMT in different ways, transforming growth factor-β1 is the most potent inducer that is capable of initiating and completing the entire EMT course, whereas hepatocyte growth factor and bone morphogenetic protein-7 act as EMT inhibitors both in vitro and in vivo [17]. Other promoters of kidney fibrosis after AKI include kidney injury molecule-1, an epithelial phosphatidyserine receptor expressed transiently after AKI [18], tumour necrosis factor-like weak inducer of apoptosis a regulator of apoptosis, proliferation and inflammation in renal epithelial cells [19], epidermal growth factor which can favour the production of profibrogenetic factors [20].

There is general agreement that the combination of DGF with acute rejection is associated with a poorer long-term graft survival in comparison with DGF without rejection [21]. Apart from the reasons reported above, it should be reminded that the endothelial cells are a privileged target of both DGF
and rejection. The endothelial reaction to any type of injury consists of remodelling of the vascular wall. This active process involves cell growth, cell death, cell migration and degradation or production of cellular matrix. These changes eventually result in intimal accumulation of smooth muscle-like cells and associated extracellular matrix, medial smooth muscle cell degeneration, adventitial fibrosis and compromised luminal flow (Figure 2).

From a practical point of view, the levels of serum creatinine after recovery from DGF are probably the best markers for long-term prognosis. Transplant patients showing only a partial recovery of graft function after DGF are at risk of deleterious long-term consequences. Actually, a poor graft function at discharge is an independent variable associated with a poor long-term outcome of the transplanted kidney [22].

**INNATE IMMUNITY ACTIVATION**

Innate immunity is an ancestral system that provides the first line of defence against invading micro-organisms. Experimental and clinical studies showed that innate immunity is deeply involved also in alloimmunity. There are numerous cells (polymorphonuclear cells, mast cells, phagocytic cells, natural killer cells and γδ cells) and mechanisms (anatomical barriers, inflammation and coagulation) involved in the innate immunity. Among them, three actors play a crucial role in organ transplantation: toll-like receptors (TLRs), dendritic cells (DCs) and the complement system.

TLRs are small proteins that recognize pathogen-associated molecules. Some TLRs (TLR2, TLR4 and TLR5) are on the outer cell membrane, while TLR3, TLR7 and TLR9 are on endosomal membrane and the remaining TLRs are in the cytoplasm. When activated, TLRs recruit adapter molecules within the cytoplasm. These adapters activate a cascade of kinases that amplify the signal leading to activation of transcription factors, such as nuclear factor-kinase B (NF-kB), mitogen-activated protein-3 and interferon regulator 3. These transcription factors, through the mediation of microRNA, can induce or suppress genes that orchestrate the inflammatory response. During an infection, pathogen-associated molecular patterns (PAMPs) released by bacteria or viruses are recognized by TLRs that activate inflammatory cells, such as polymorphonuclear cells, monocyte/macrophages, natural killers, and humoral mediators such as complement and short-term pentraxins mitochondria are also an important component of the innate immunity and antibacterial responses. A subset of TLRs (TLR1, TLR2 and TLR4) can recruit mitochondria to macrophage phagosomes and induce the generation of mitochondrial ROS [23].

TLRs can be engaged not only by microbial-associated molecular patterns but also by endogenous molecules called danger-associated molecular patterns (DAMPs) that are released by cells damaged or killed by injury or disease [24]. When released into extracellular space, DAMPs are recognized as danger signals and activate TLRs in a fashion analogous to PAMPs (Figure 3).

In the setting of organ transplantation, TLRs play an important role in the maladaptive response to AKI [25]. The tubular up-regulation of TLRs caused by AKI results in exaggerated production of pro-inflammatory, pro-fibrotic and vasoconstrictor genes that can eventually lead to tubular atrophy, interstitial fibrosis and progressive renal injury [26]. There is also experimental and clinical evidence that TLR signalling is involved in the immune recognition of allografts [27]. After recognizing the specific DAMPs, TLRs not only trigger an inflammatory response by the cells and mediators of the innate immunity but can also alarm the DCs.

DCs derive from the differentiation of haematopoietic stem cells and are recruited to peripheral tissues [28]. Immature DCs are tolerogenic, but once they come into contact with a presentable antigen in an inflammatory environment DCs become mature, capture and process the antigen by degrading its proteins into small pieces and through lymphatic vessels migrate out of the graft to lymph nodes, where they present the antigen to immunocompetent cells (Figure 4). In the lymphatic system, mature DCs up-regulate cell-surface co-receptors such as CD80 (also called B7.1), CD86 (or B7.2) and CD40 that enhance the activation of T and B cells [29]. DCs can act as antigen-presenting cells (APCs) by presenting epitopes to T cells via three mechanisms: (i) a direct pathway, in which recipient T cells recognize intact donor antigens of the major histocompatibility complex (MHC) presented by donor DCs; (ii) an indirect pathway, whereby T cells recognize peptides derived from allogenic MHC proteins presented by self-APC; (iii) a semidirect pathway in which the recognition of donor MHC molecules is transferred from donor DCs to recipient APC [30].

A central facet of innate immunity is the complement system. Three biochemical pathways can activate the complement cascade: classical pathway, alternative and lectin-mannose. Each of these pathways may be activated by different mechanisms and can modulate the alloresponse in different directions. DAMPs may activate the classical pathway by binding to C1q (classical pathway), or the alternative pathway by binding to C3, or the lectin-mannose pathway by binding to mannose-
The interaction between T and B cells can generate different alloimmune responses. The strict stimulation signal). T cell receptor and co-stimulation molecules (CD80 also called B7-1; CD86 also called B7-2) and CD28 located on the surface of T cell (co-stimulatory signal). T cell receptor and co-stimulation molecules generate different signals of transduction that activate T cell. The strict interaction between T and B cells can generate different alloimmune responses.

**FIGURE 3**: A schematic view of innate inflammatory response. The recognition receptor pattern of innate immunity is mainly composed by TLRs, but also the intracellular receptors, NOD-like receptors and RIG-I receptors, may contribute in recognizing the PAMPs or DAMPs, as in the case of endogenous substances release by tubular and endothelial cells injured by ischaemia reperfusion. TLRs recruit in the cytoplasm adaptor molecules (MyD88, TIRAP, TRIF, Tram), that activate a number of kinases (IRAK1, IRAK4, TBK1, IKK) which amplify the signal and transmit it to transcription factors NF-kB, MAP-3 and IFR3 that encode the genes regulating the inflammatory cells. Myd88, myeloid differentiation primary response 88. TIRAP, total interleukin 1 receptor protein. Trif (also called TICAM-1), the toll-IL-1 receptor (TIR) domain-containing adaptor molecule-1. TRAM, Trif-related adaptor molecule. IRAK, interleukin-1 receptor associated kinase. TBK1, TANK-binding kinase 1, IKK, I kB, MAP-3, kinase myogen-activated protein-3; IFR3, interferon regulator 3, NF-kB, nuclear factor kinase B.

**FIGURE 4**: In the presence of an inflammatory milieu, dendritic cells become mature, intercept the antigen (Ag) and migrate to lymph nodes where they present the antigen to T cells. The activation of T cell requires two steps: (i) The contact between the antigen and the specific T receptor. (ii) The contact between adhesion molecules on the surface of the antigen-presenting cell (CD80 also called B7-1; CD86 also called B7-2) and CD28 located on the surface of T cell (co-stimulation signal). T cell receptor and co-stimulation molecules generate different signals of transduction that activate T cell. The strict interaction between T and B cells can generate different alloimmune responses.

**IS IT POSSIBLE TO PREVENT IRI AND ITS CONSEQUENCES?**

**Management of the donor**

Efforts to decrease the impact of IRI during the transplant process include an optimal management of deceased donors, accurate surgical technique, minimizing cold ischaemia time, optimizing allograft perfusion during intra- and post-operative periods.

The goals of management of donor are to achieve normal volemia, maintain blood pressure and optimize cardiac output so as to obtain adequate perfusion pressure and blood flow with the use of the least amount of vaso-active drug support. To restore a normal volemia, hypotonic saline solutions should be preferred since most donors show hypernatremia. Sodium bicarbonate should be added to correct the concomitant acidosis, and packed red blood cells should be transfused to keep haemoglobin levels >10 g/dL. Antiuretic hormone should be considered in donors with diabetes insipidus, and insulin is required in case of hyperglycaemia caused by too generous administration of glucose solutions [35].

Donor pre-treatments have been investigated in experimental studies. Ischaemic preconditioning consisting of 15 min of warm ischaemia and 10 min of reperfusion could protect from warm and cold ischaemia in a murine model [36]. Remote ischaemic preconditioning by inducing a brief ischaemia of the limb may also reduce the impact of IRI [37]. The administration of thymoglobulins to rats with brain death reduced the expression of pro-inflammatory cytokines and ameliorated renal damage [38]. Supplementation of Klotho, a transmembrane protein with pleiotropic functions, may protect from IRI and suppress fibrosis [39].
**Management of the recipient**

If the recipient needs to be submitted to haemodialysis before transplantation, it is recommended to avoid a strong dehydration and to wait some hours before transplantation in order to avoid hypovolemia and a hypercoagulable rebound. The central vein pressure of the recipient should be maintained in a supranormal range in order to allow a satisfactory perfusion of the transplanted kidney. To prevent AKI, the European Renal Best Practice guidelines recommend that in the absence of haemorrhagic shock, isotonic crystalloids rather than colloids (albumin or starches) should be used as initial management for expansion of intravascular volume in patients at risk for AKI [40]. Plasma expanders or hypertonic mannitol may be administered immediately before the vascular connection. The use of nephrotoxic drugs, such as aminoglycoside, fluoroquinolones, amphotericin B, foscarnet should be avoided, whenever possible. In patients at higher risk of DGF, such as old recipients of old kidneys, the administration of calcineurin inhibitors may be delayed until serum creatinine falls under 2.0–2.5 mg/dL [41].

**Vasodilators and hormones**

A meta-analysis of randomized controlled trials showed that calcium-channel blockers given in the peri-operative period may reduce the incidence of AKI post-transplantation [42]. However, this data should be treated with caution due to the heterogeneity of the trials and the possibility that some calcium-channel blockers can increase the blood levels of calcineurin inhibitors. Fenoldopam, Atrial Natriuretic Peptide (ANP), Brain Natriuretic Peptide (BNP) and Dopamine proved to be helpful in reducing the risk of AKI in experimental models. A review made in patients who received cardiovascular surgery reported that Fenoldopam and ANP reduced the need for renal replacement therapy by 5 and 3.5%, respectively. BNP resulted in a 10% reduction in the incidence of AKI, while Dopamine caused a significant reduction in creatinine clearance, ~4.5 mL/min [43]. Only small inconclusive trials with these agents have been done in kidney transplantation. Thyroid hormone therapy has also been used but a systematic review of the available trials concluded that this therapy may be associated with worse outcomes for patients with established AKI [44].

**Antioxidant therapy**

Intravenous SOD administered during transplantation significantly reduced the incidence of acute rejection and improved the 4-year graft survival in recipients of deceased donor kidneys [45]. L-Argininhould also improve kidney graft function, although the effect was mainly observed with kidney coming from young donors with short cold ischaemia time [46]. In a small trial, graft recipients of deceased renal donors were assigned to treatment with N-acetylcyesteine, 600 mg twice a day, or control. DGF was significantly lower among the treated group and markers of oxidative stress were significantly attenuated in treated recipients [47]. Other promising results have been obtained in experimental studies with peroxinitrite, ligustrazine, vitamin E, cell permeants SOD mimetics, nitric oxide generators such as nitrosithiols, tempol-folate and allopurinol. However, only a few studies of poor quality have been made in clinical transplantation with a few of these agents and we still lack unambiguous demonstration that pre-treatment with these antioxidants can attenuate the impact of IRI in kidney transplantation. A few investigators even reported failure in applying antioxidants to pathologies where the causal role of ROS was supposed [48].

**Storing donated kidney**

Adequate preservation of renal allografts during cold ischaemia is important for preventing DGF. For cold static storage, University of Wisconsin and Celsior are the solutions more frequently used. Pulsatile hypothermic machine perfusion is an alternative to cold storage. A systematic review and meta-analysis showed that hypothermic machine perfusion significantly reduced DGF compared with static cold storage. There was no difference in primary non-function, acute rejection, long-term renal function or patient survival. A difference in renal graft survival was uncertain [49].

**Inhibition of innate inflammatory response**

A different approach is addressed to inhibit the signals leading to inflammation. The present focus of many investigations is to understand the complex regulation of TLR signalling, including post-transcriptional regulation such as ubiquitation, phosphorylation and mRNA stability, and to recognize the genes that exert the spatial, and in some cases temporal, regulation of the innate immunity pathways.

Experimental studies showed that it is possible to prevent the activation of the innate immunity, by inhibiting TLR2 which is expressed on tubular epithelial cells together with TLR4 and can contribute with complement in inducing the production of inflammatory cytokines [50]. Depletion of TLR2 with a new monoclonal antibody could significantly reduce the impact of IRI in some models of myocardial IRI [51], suggesting possible application also in kidney transplantation. Other possible targets for future attempts may be represented by the adaptive molecule MYD88 [52], natural killer cells [53] and inflammasomes [54].

Inhibition of C5a and the membrane attack complex C5b-9 could be another target. Eculizumab, a humanized monoclonal antibody directed against the C5 component of the complement cascade, has been used in renal transplantation to treat atypical haemolytic-uraemic syndrome and antibody-mediated rejection. Theoretically, a short course of eculizumab could also be used for preventing IRI. Studies to evaluate the role of eculizumab in the prevention and treatment of IRI in kidney allografts are currently ongoing (ClinicalTrials.gov Identifier: NCT01756508, NCT01403389).

**Manipulation of DCs**

Manipulation of DCs is another promising approach. DCs have a pivotal role in the immune response as they can operate as a bridge between innate and adaptive immunity. Some immunosuppressive drugs currently used in organ transplantation may interfere with the function of DCs. Rabbit anti-thymocyte globulins inhibit DCs function [55] and promote expansion of regulatory T cells [56]. The inhibitors of mTOR
may induce resistance to phenotypic maturation of DCs induced by inflammation and may facilitate the production of regulatory tolerogenic DCs [57]. On the other hand, inhibition of mTOR promotes pro-inflammatory cytokines such as IL-12 and IL-1β and inhibits the anti-inflammatory cytokine IL-10, suggesting that anti-mTOR drugs can regulate both innate and adaptive immune responses.

NF-κB pathway is responsible for inflammation and DC maturation. Thus, inhibition of this transcription factor may affect the function of DCs. Apart from high-dose corticosteroids and aspirin, laquinimod, an experimental immunomodulator, seems to be particularly effective in inhibiting NF-κB. In an animal model of relapsing-remitting multiple sclerosis, laquinimod inhibited NF-κB pathway and modified the maturation and function of DCs [58]. Plasmacytoid DCs are a specialized subset of DCs which produce type I interferon in response to viral infection. Differently from conventional DCs, plasmacytoid DCs alter co-stimulatory molecule expression and reduce allostimulatory capacity when interacting with T cells. Thus, plasmacytoid DCs play an important role in the regulation of mucosal immunity. Moreover, this phenotype favours the generation of alloantigen-specific regulatory CD4(+) or CD8(+) T cells, critical to the development of graft tolerance [59]. Isolation and in vitro generation of gene-manipulated human 'plasmacytoid DCs may represent a further target to prevent the consequences of IRI and favour an operational tolerance in kidney transplantation'.

**CONCLUSIONS**

IRI is an inevitable event after deceased donor transplant and can heavily influence both the early and the late function of a kidney allograft. At least 445 studies on IRI have been registered in ClinicalTrials.gov. Hopefully, some of these investigations could better clarify the complex pathogenesis and suggest some effective treatment of IRI. At present, however, we lack specific therapies to prevent IRI in clinical transplantation.

The current treatment is aimed to contrast the main consequences of IRI, i.e. DGF and activation of innate immunity. Prevention of DGF mainly rests on pre-transplant treatment of the donor, perfusion of the kidney with intracellular-like solutions and correct hydration of the recipient. The use of pulsatile perfusion machine may significantly reduce the DGF in comparison with cold static storage and may increase the utilization of non-heart-beating donors. Promising findings obtained in small animal models with vasodilators, hormones and antioxidant agents still await for more solid data before being recommended in clinical transplantation.

Interfering with innate immunity is difficult. Intravenous methylprednisolone pulses might inhibit inflammatory genes and anti-thymocyte globulins may inhibit DCs function and promote expansion of regulatory T cells. However, the real impact of these agents in preventing the effects of innate immunity is doubtful. A promising approach might rest on Eculizumab, but the cost of this agent is extremely elevated and no data about its efficacy in preventing IRI or its effects is available.

In summary, at present the possibility of interfering with IRI is still limited to time-honoured preventive measures. In the near future, a number of new therapeutic strategies currently under investigation will have the potential to interfere with IRI and improve clinical outcomes in renal transplant recipients.

**CONFLICT OF INTEREST STATEMENT**

C.P. has been consultant of Novartis Italy until December 2011. In the last 2 years, he received honoraria for invited lectures from Novartis, University of Calgary (Canada), North Shore Hospital of New York (USA). The results presented in this paper have not been published previously in whole or part, except in abstract format.

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