Long-term effects of acute ischemia and reperfusion injury

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Long-term effects of acute ischemia and reperfusion injury. Ischemia reperfusion (I/R) injury plays a major role in delayed graft function and long-term changes after kidney transplantation. By using different therapeutic strategies to prevent I/R injury in rat models of kidney transplantation we studied relationships between inflammatory cell arrival and adhesion molecule expression. In other rat models for acute renal failure we investigated the effect of up-regulation of protective genes such as heme oxygenase-1 (HO-1) on infiltrating cells, showing that infiltrating cells also contribute to beneficial effects. In order to gain more insight into the complex mechanisms of long-term changes after kidney transplantation, we started a protocol biopsy program to study histologic changes 6, 12, and 26 weeks after transplantation. The following article clarifies some of the complex mechanisms contributing to long-term changes caused by I/R injury.

The last years, it has become evident that the cellular and molecular mechanisms during ischemia and reperfusion resemble an acute inflammatory response. Acute ischemia leads to the activation of the endothelium with an increase in permeability and expression of different adhesion molecules [1, 2]. These molecules are crucial for the recruitment and infiltration of effector cells into the postischemic tissue [3]. Transcription factors such as nuclear factor-κB (NF-κB) are induced and activated leading to an enhanced expression of inflammatory genes. The endothelial cells lose their antiadhesive properties and develop a thrombogenic and adhesive surface. On reperfusion, the ischemia-primed endothelial cells are prone to leukocyte and platelet adhesion, thereby increasing endothelial cell permeability and cell activation. The adherent leukocytes release reactive oxygen species (ROS) and a variety of cytokines, enhancing the inflammatory reaction. Subsequently, the leukocytes transmigrate and enter the subendothelial space. The acute inflammatory response then leads to organ dysfunction and, eventually, to organ failure. Eventually, the ischemia/reperfusion (I/R) injury is resolved and organ function restored [4]. However, after initial recovery and a period of relative quiescence, proteinuria may develop and progressive morphologic changes begin, including glomerulosclerosis, arterial obliteration, and interstitial fibrosis. These phenomena are accompanied by a reexpression of adhesion molecules, progressive macrophage infiltration and their associated products, particularly interleukin (IL)-1, tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), and inducible nitric oxide synthase (iNOS). Monocyte chemoattractant protein-1 (MCP-1) is subsequently up-regulated, with a dramatic increase in infiltrating cells.

To what extent the acute cellular alterations persist long-term and affect organ function at later time points remain unclear. Long-term effects of I/R injury have been of special interest in kidney transplantation [5, 6]. After removal from the donor the organs rapidly develop signs of ischemia. Despite adequate cooling and the use of preservation fluids, the ischemia leads to the above-described molecular changes in the vasculature and the interstitium of the organ. Upon reperfusion in the recipient an acute inflammatory response is induced which may lead to acute renal failure (ARF), the so-called delayed graft function. Numerous strategies have been tested to prevent I/R injury and to ameliorate immediate graft function. Most studies have supported the concept that inflammatory mechanisms are important mediators of I/R injury and have shown that inhibition of adhesion molecule expression, decrease of endothelial cell activation or prevention of leukocytes adhesion prevent or ameliorate delayed graft function. However, it is less clear whether and how these acute effects may affect the long-term function of the transplanted kidney and whether I/R injury may play a role in the development of chronic allograft nephropathy.

I/R INJURY IS A STATE OF INFLAMMATION AND MAY PERSIST

Transplanted kidneys with prolonged cold or warm ischemia times are more susceptible to short-term or long-term deterioration. Clarifying the pathophysiology of I/R injury and developing new preventive strategies are highly important. In native organs, numerous experimental and clinical studies showed that I/R constitutes an acute inflammatory process, involving cell surface

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adhesion molecule expression. In transplanted organs, the same mechanisms are also involved in subsequently initiating the rejection process [4]. Although numerous studies have addressed anti-adhesion strategies in rodent models of I/R [8, 9], the sequence in which different cell subsets arrive and their time course in relationship to I/R have not been extensively studied. We tested the hypothesis that I/R is solely responsible for the activation of the adhesion cascade and that alloantigen-dependent mechanisms are not primarily operative in the early post-transplant period [20]. We addressed differences between alloantigen-dependent and alloantigen-non-dependent injury by employing syngeneic (Lewis-to-Lewis), nonimmunosuppressed, and cyclosporine A (CsA)-immunosuppressed allogeneic (Fischer-to-Lewis) models of rat renal transplantation. We investigated the sequence in which different cell subsets arrived in the I/R-damaged transplanted kidney. Finally, we studied the relationship between inflammatory cell arrival and adhesion molecule expression on endothelium and leukocytes in the time framework of I/R, as well as deterioration and graft recovery [20]. The affected vessels expressed more vascular cell adhesion molecule-1 (VCAM-1) and dense monocytic infiltrates.

ACUTE PREVENTION OF LEUKOCYTE ADHESION AND LONG-TERM CHANGES

Others and we have recently examined the long-term effects of an antiadhesive strategy in the prevention of ARF in experimental kidney transplantation and observed persistence of macrophage infiltration, induction of major histocompatability complex (MHC) class molecules and increased interstitial fibrosis 4 weeks after acute injury [2, 8, 9]. Therapeutic strategies, which ameliorate endothelial cell dysfunction and cell infiltration, seem to prevent the long-term damage after I/R injury [9]. We were able to demonstrate that antisense directed against intercellular adhesion molecule-1 (ICAM-1) markedly attenuated acute reperfusion injury in an autotransplanted model and had a profound effect on long-term outcome. Rats with allograft control kidneys developed proteinuria with gradual renal failure leading to death by week 12. We observed infiltrating lymphocytes and macrophages, tubular atrophy, interstitial fibrosis, arteriosclerosis, and glomerular atrophy, sclerosis, and obsolescence. Similar chronic changes after I/R injury have been found by others [10, 21]. A possible explanation of the long-term effects of I/R is the acute injury to the glomeruli. I/R injury most likely reduced the nephron mass in our rats, not only through adhesion molecule-related events but also by ischemia-induced tubular necrosis. The effect of reduced nephron mass has been emphasized in both animal experiments [11] and observations in transplanted patients [12].

However, from our observation we would favor a role for monocytes/macrophages in the chronic response to acute I/R injury. A major finding in our studies was the massive infiltration of monocytes. Vehicle-treated kidneys showed marked monocytic infiltration in the interstitium and around blood vessels. In contrast, kidneys from animals with an antisense-treated kidney show only occasional monocytes in the glomeruli and interstitium. Monocytes are recruited to the tissue during I/R injury and comprise up to 60% of the cell infiltrate. These monocytes are recruited by chemokines from activated endothelium such as MCP-1 and regulated upon activation, normal T-cell expressed and secreted (RANTES) after tissue infiltration monocytes mature and differentiate into resident macrophages. In addition, it has been shown that tissue macrophages can proliferate, thereby amplifying and prolonging the local inflammatory response. An important growth factor for macrophage proliferation is macrophage-colony-stimulating factor (M-CSF) [13]. The receptor for this growth factor is encoded by the proto-oncogene c-fms. In an earlier study we have investigated the expression of c-fms in areas of cardiac fibrosis [14]. We reasoned that the appearance of c-fms might be associated with an increase in monocytes. With in situ hybridization, we were able to show that the c-fms expression did not emanate from the cardiac myocytes themselves, but rather from perivascular areas and sites between the cardiac myocytes. Histochemical staining with a specific monocyte marker antibody corroborated the presence of increased monocytes at these sites. Infiltrating monocytes and macrophages are capable of elaborating cytokines, including platelet-derived growth factor (PDGF). Expression of c-fms is a marker of monocyte differentiation [15]. Our findings suggest that increased c-fms expression not only demonstrates the presence of monocytes but also implies that monocytes display a differentiated phenotype with increased cytokine receptor expression. We suggested that an increase in this product could result in proliferation of monocytes within the perivascular areas of the myocardium. The monocytes may be integrally involved in the scarring process. The early interstitial changes in the heart resemble strongly the interstitial and vascular alterations in the kidneys of transplants. We observed increased interstitial deposition of the same collagens both in renal and cardiac fibrosis. A progressive infiltration of monocytes and macrophages was identified in the walls of blood vessels and the interstitium. We have not elucidated the initial signal for monocyte infiltration nor their subsequent behavior. The signaling may involve the renin-angiotensin-aldosterone system. Further support for such a hypothesis and a role of macrophage survival and proliferation comes from a recent study by Jose et al [16] who observed organ protection after blockade of the M-CSF receptor in transplanted animals.

PROTECTIVE ROLE OF INFILTRATING CELLS

Macrophage infiltration seems not only associated with tissue damage. It is well established that the cellular infiltrate after I/R injury consists of several subsets of leukocytes, which may have deleterious or beneficial effects on the chronic inflammatory process. We recently identified one of the possible beneficial effects of infiltrating macrophages. We addressed the question whether acute treatment during I/R may influence the expression of protective genes such as heme oxygenase-1 (HO-1). The HO-1 system is one of the most important cytoprotective mechanisms. The HO-1 isoform is also known as heat-shock protein 32 (HSP32). Its expression is relatively low under physiologic conditions but strongly induced in hypoxia, ischemia, inflammation, or radiation [17]. Since we had previously demonstrated an anti-inflammatory effect of statins in an animal model of renal injury [18], we investigated the effects of a statin on acute I/R injury [19]. We observed that three-day statin treatment significantly ameliorated the decrease in renal function with postischemic acute tubular necrosis and considerably limited the structural damage after ischemia. Histologically, we observed that statin treatment reduced damage in the proximal tubules in the outer medullary stripe, the area that is most susceptible to hypoperfusion and hypoxia. Statin treatment clearly reduced the morphologic damage. Inflammatory reactions attributable to I/R injury are characterized by leukocyte infiltration. In postischemic ARF, the majority of infiltrating cells are endothelin-1 (ED-1)–positive monocytes and macrophages [20]. We observed that the statin reduced ED-1–positive cell infiltration by >50%, compared with no treatment. Inflammatory cell infiltration in I/R injury is accompanied by significant up-regulation of adhesion molecules [20]. We observed that activation of the adhesion cascade, as reflected by up-regulation of ICAM-1 on the endothelium of arteries and in the perivascular space, was inhibited by the statin. The up-regulation of ICAM-1 expression in glomeruli, the periglomerular area, and the peritubular interstitium was also prevented. These findings indicated that statins mostly act through the inhibition of macrophage infiltration. However, when we investigated the expression of the "protective" gene HO-1, a significant up-regulation of this gene both on the RNA and protein level was found. Further analysis of HO-1 expression showed an increase of HO-1 in larger vessels and in the interstitial area. Most of the infiltrated macrophages showed a significant increase in HO-1 while lymphocytes were not different from untreated control animals. This finding indicates that the treatment with a statin did not only reduce the infiltration of monocytes/macrophages but, at the same time, induces "protective" genes which may alter cytokine and protease expression in the infiltrated cells. Such a hypothesis is further supported by our finding that statins may in vitro induce HO-1 expression.

We also observed less fibrosis after treatment with a statin. Fibronectin is a major glycoprotein in plasma and in the extracellular matrix. It plays a role as a chemoattractant for several cell types and generates a scaffold to which other matrix components can attach. In response to I/R injury, fibronectin is markedly up-regulated in the renal interstitium [21]. We observed that cerivastatin effectively blocked the increased expression of fibronectin in the tubulointerstitium of the outer medullary stripe. We also examined collagen IV expression, because abnormal collagen turnover is a major histologic feature in many forms of renal disease. Collagen IV expression is also predictive for long-term allograft survival. Collagen IV occurs in the renal interstitium, Bowman's capsule, and tubular basement membranes [22]. We observed that the increased collagen IV deposition in the peritubular interstitium after I/R injury was decreased with statin treatment. We found that I/R is ameliorated by treatment with a statin and that this strategy not only reduces endothelial cell function and leukocyte infiltration but also the expression of HO-1 in a subset of infiltrating cells.

PROTOCOL BIOPSIES AS A STRATEGY TO ANALYZE THE MECHANISMS OF I/R-INDUCED CHRONIC CHANGES

In order to gain more insight into the cellular and molecular changes in the early period after transplantation and analyze the relationship between I/R and chronic changes protocol biopsies 6 weeks and 3 and 6 months after renal transplantation were analyzed. Chronic allograft nephropathy (CAN) leads to chronic allograft dysfunction and is the most prevalent cause of late renal graft loss. CAN probably starts early after transplantation, in correlation with donor conditions, such as donor age, brain death, or I/R injury [22–25], but is found particularly in the case of early rejections [26–28]. In December 2000, we therefore started a renal transplant protocol biopsy program, with the intention of (*1*) detecting and treating clinically silent acute rejection episodes [29] and (*2*) identifying early mechanisms of CAN.

The protocol biopsies are carried out 6, 12, and 26 weeks after renal transplantation as an outpatient procedure. Biopsies are performed in the morning using an 18-gauge automated biopsy needle and the patient is kept in hospital for 4 hours bed-rest with blood pressure and urine control and carrying out an ultrasound of the kidney before and after biopsy. The histology of the biopsies was evaluated according to the Banff classification system [30].

Since December 2000 we have performed 228 transplant biopsies in 108 patients [biopsy 1 ($N = 93$), biopsy 2 ($N = 82$), and biopsy 3 ($N = 53$)]. Fourteen patients (13%) had several transplants, 14 patients (13%) had a combined pancreas-kidney transplant and 16 patients (15%) had a transplant from a living donor. The patient acceptance rate of the program was 96%. Complications

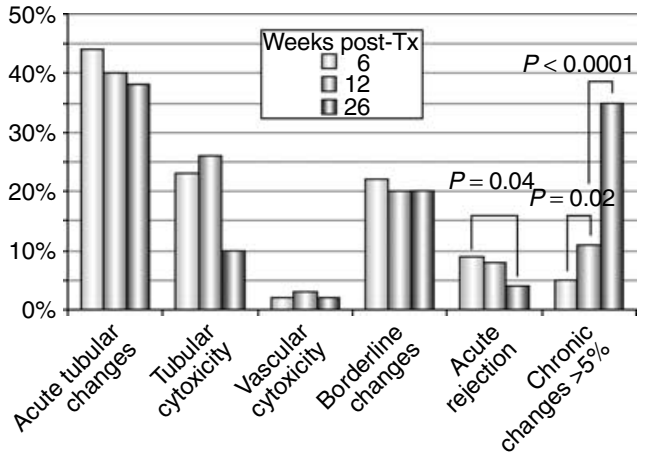

Fig. 1. Histopathologic findings in relation to time after renal transplantation (Tx).

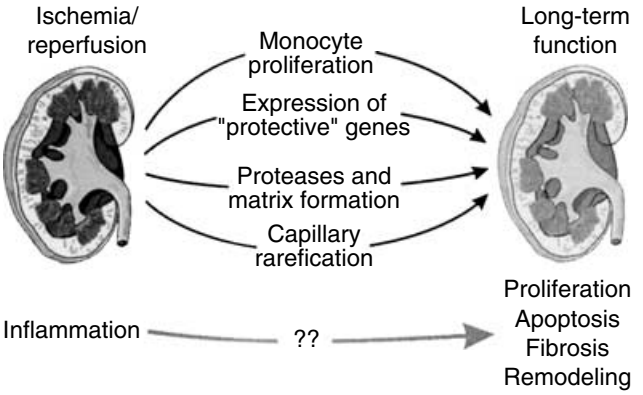

Fig. 2. Possible mechanisms of ischemic/reperfusion (I/R)-induced chronic changes.

of the 228 biopsies were a vasovagal reaction in three cases with sustained bradycardia or vomiting, macrohematuria in four cases, a perirenal hematoma in ultrasound in nine cases, and a small arteriovenous fistula in 23 cases. Hospitalization of the patient due to a biopsy complication was necessary in four cases. The histologic findings in relation to time after renal transplantation are shown in Figure 1. Acute rejections were most often seen in biopsy 1, chronic changes most often in biopsy 3 (Fig. 1). Chronic changes such as interstitial fibrosis indicating CAN were already detected in 52% of biopsies after 6 months. These patients also had a small but significant increase in serum creatinine. We then tested whether cold ischemia time, delayed graft function, donor age, and other well-established risk factors were related to these early interstitial changes. So far, no statistical significant correlation has been observed. These observations indicate that the mechanisms, which induce interstitial fibrosis in the months after I/R injury, are still incompletely understood. We propose that the chronic effects of I/R depend on the individual response of the kidney to the insult. Important cellular mechanisms may be the balance between damaging genes such as oxidases and proteases and protective genes such as HO-1 and VEGF.

FUTURE DIRECTIONS

Our studies indicate that the cellular and molecular mechanisms, which persist in the months after I/R injury, are still unclear. Obviously, the extent of the intitial injury (i.e., the degree of necrosis and apoptosis) plays an important role. Other possible cellular mechanisms are shown in Figure 2. Monocyte infiltration seems to be a prerequisite of fibrosis and subsequent deterioration. An analysis of the macrophage subsets in the graft and their gene expression pattern is an important future goal. However, which mechanisms are responsible for monocyte survival, which factors are responsible for differentiation into macrophages and whether in situ proliferation of the monocytes is important in the pathogenesis of chronic nephropathy have to be analyzed further. The hallmark of chronic changes after ischemia and reperfusion injury is fibrosis. It seems that the balance between protease activity and expression of matrix molecules is important. We recently began to investigate the role of plasminogen activators in this context [31]. In animal experiments, it has been shown that plasminogen activator plays an important role in vascular remodelling and fibrosis after transplantation [32]. However, with regard to monocytic expression of urokinase-type plasminogen activator (uPA) and its receptor we have found a wide variation of individual responses in our protocol biopsies. This observation supports the concept that the individual cellular and molecular response to the respective injury plays an important role in the pathogenesis of CAN. Analysis of individual patterns of gene expression using microarrays may be a useful approach to define these "response" patterns.

Structural changes in the kidney after the initial injury may contribute to the chronic fibrosis and deterioration. The loss of glomeruli and subsequent increase in "renal workload" has been proposed by others [11, 12]. However, capillaries in other areas of the kidney may also be affected by I/R injury. A rarefication of capillaries has recently been suggested as a critical step in the pathogenesis of chronic renal disease [33].

It seems unlikely that only one of these mechanisms is responsible for the induction of fibrosis in kidneys after I/R injury [34]. The magnitude and the type of injury play an important role. It will also be necessary to characterize the relationship between types of tissue injury and to find out how different types of injury influence each other. It is also important to realize the time of the respective injury. Chronic or repetitive exposure to injury seems to have a deleterious effect on long-term organ function. This conceptual approach is especially important in kidney transplantation because of the combination of immunologic and nonimmunologic injury as well as the effect of the immunosuppressive therapy [35] (Fig. 3). In addition, individual response patterns may be important.

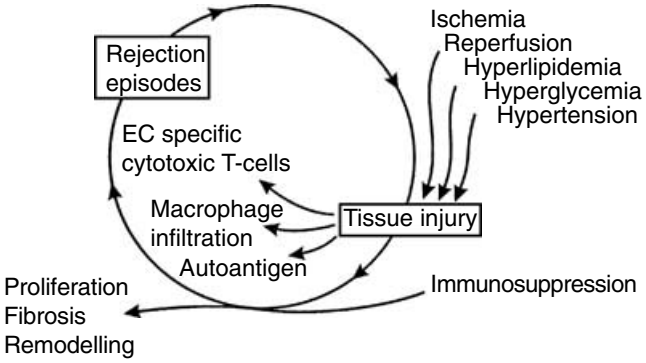

Fig. 3. Relationship between immunologic and nonimmunologic mechanisms in the pathogenesis of chronic allograft nephropathy (CAN). EC is endothelial cell.

Some kidneys (or some recipients) may be more resistant to injury than others. Identification of gene expression patterns over time may be necessary to characterize the molecular response of each patient and to "tailor-made" therapeutic strategy.

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