Cold ischemia augments allogeneic-mediated injury in rat kidney allografts

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Background. Some clinical studies demonstrate that kidney grafts with prolonged cold ischemia experience early acute rejection more often than those with minimal ischemia. The mechanism, however, is putative. Therefore, the aim of this study was to unravel the impact of ischemia on the immune response in rat kidney allografts compared with that in isografts.

Methods. To induce ischemic injury, donor kidneys were preserved for 24 hours in 4°C University of Wisconsin solution before transplantation. No immunosuppression was administered. The histomorphology according to the BANFF criteria for acute rejection and infiltrating cells were assessed at days 1, 2, 3, 4, 6, and 8 post-transplantation.

Results. In allografts, exposure of the kidney to ischemia led to a significantly earlier onset of interstitial cell infiltration and tubulitis compared with nonischemic allografts. The BANFF score of interstitial cell infiltration was 1 ± 0 vs. 0.25 ± 0.29 at day 3 and 2 ± 0 vs. 1.25 ± 0.25 at day 4. In contrast, in isografts, the effect of ischemia on the histology was not significant. From day 6, the histologic differences between ischemic and nonischemic grafts disappeared. Ischemia led to a more intense expression of P-selectin (day 1), intercellular adhesion molecule-1 (ICAM-1; day 2), and major histocompatibility complex (MHC) class II on endothelium and proximal tubular cells (day 2) in both allografts and isografts. Concurrently with the up-regulated ICAM-1 and MHC expression, significantly more CD4+ cells and macrophages infiltrated the ischemic allografts at days 2 and 3 and the ischemic isografts at day 4. Importantly, the influx of these cells after ischemia was significantly greater in allografts than in isografts.

Conclusions. Cold ischemia augments allogeneic-mediated cell infiltration in rat kidney allografts. The earlier onset of acute rejection in 24-hour cold preserved allografts may be prevented by better preservation or treatment using tailored immunosuppression.

Key words: acute rejection, kidney transplantation, isograft, inflammatory response, major histocompatibility complex.

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Methods

Animals

Male rats of the inbred Brown Norway (BN) (RTI^) and WAG/Rij (RTI) strains were purchased from Harlan (Horst, The Netherlands). The rats weighed 200 to 250 g and were two to three months of age at the beginning of the experiment. All animals were kept under standard conditions and were given access to standard commercial rat chow (AM II; Hope Farms, Woerden, The Netherlands) and tap water acidified to pH 3 ad
libitum. The experimental protocols were approved by the committee on animal research of the Erasmus University (Rotterdam, The Netherlands).

**Kidney transplantation**

Kidney transplantation was performed using a modification of the technique described by Fisher and Lee [11]. The animals were anesthetized with ether. After an intravenous injection of heparin (100 IU), the left donor kidney was flushed in situ via the aorta with 5 mL University of Wisconsin (UW) solution of 4°C at a rate of 2 mL/min. The kidney was excised and stored in UW solution prior to implantation. In the recipient, the kidney graft was transplanted heterotopically; donor renal artery and vein were anastomosed end to side to recipient aorta and vena cava, respectively, using continuous 9-0 prolene sutures. During surgery, the graft was wrapped in gauze moisturized with 4°C phosphate-buffered saline (PBS). The perioperative ischemic time was 30 minutes. After revascularization, the ureter was anastomosed end to end to the distal third part of the recipient’s ureter using single 11-0 prolene sutures. The ipsilateral native kidney was removed at the time of transplant, whereas the contralateral one remained untouched until sacrifice. The mean survival time for kidney allografts without immunosuppression in a bilaterally nephrectomized recipient in this strain combination is 10 ± 2 days.

**Cold ischemia period**

The donor kidney was stored at 4°C for either 10 minutes, which is the minimal time in which the recipient is anesthetized and prepared for implantation of the graft, or 24 hours. The 10-minute cold ischemia subsequently will be referred to as nonischemia.

**Experimental groups**

The impact of prolonged cold ischemia on the development of acute rejection was studied in the BN-WAG donor–host combination without the use of immunosuppression. Nonischemic BN-WAG allografts and ischemic and nonischemic WAG-WAG isografts served as controls to determine the relationship between ischemia and allogenicity.

Representative specimens of kidney grafts were removed and snap frozen in liquid nitrogen for immunohistochemistry. Kidney specimens were also placed in 3.6% buffered formaldehyde for histomorphology.

Morphological and immunohistological evaluation was performed at days 1, 2, 3, 4, 6, and 8 post-transplantation (N = 5 animals/group/time point for allografts and N = 3 animals/group/time point for isografts).

**Macroscopy**

At the time of sacrifice, the ureter was inspected for its diameter. The kidney transplants were examined for signs of acute rejection: swelling, hemorrhage, and infarction. Kidney grafts with early hydronephrosis or pyelonephritis were excluded from the study.

**Histology**

Paraffin-embedded 3 μm sections were cut, and hematoxylin and eosin, periodic acid-Schiff (PAS), and Jones-staining were performed. Histologic analysis was performed using the BANFF criteria for acute rejection [12, 13] in a blind fashion.

**Immunohistology**

Representative portions of all kidneys were stained on 5 μm cryostat sections by a three-layer immunoperoxidase technique. After fixation with acetone for 10 minutes, endogenous peroxidase activity was blocked by incubation for 10 minutes in methanol/0.03% H2O2, after dehydration through graded alcohols. After rehydration, the nonspecific binding was blocked by preincubation with 10% normal rabbit serum (Dako, Copenhagen, Denmark) in PBS/bovine serum albumin 5%. This was followed by one hour of incubation with primary monoclonal antibodies (Serotec, Oxford, UK) for identification of CD45+ leukocytes (OX-1), CD4+ cells (W3/25), CD8+ cells (OX-8), monocytes/macrophages (ED-1), natural killer (NK) cells (3.2.3), MHC class II antigens (OX-6), and intercellular adhesion molecule-1 (ICAM-1). After each incubation, the slides were washed in PBS-Tween 20, 0.1%. A second layer, rabbit anti-mouse IgG (Dako), was then applied for 30 minutes and after washing, the slides were incubated with the third layer, mouse peroxidase-antiperoxidase (Dako), for 30 minutes. After washing in PBS, the reaction was developed by the addition of diaminobenzidine substrate (Dako) for eight minutes, and slides were counterstained in Mayer’s hematoxylin for 40 seconds, washed, dehydrated, and mounted. In order to stain the rabbit polyclonal antibody P-selectin (Pharmingen, San Diego, CA, USA), the preincubation was done with Normal Swine serum (Dako), and after staining, the sections were incubated with swine antirabbit IgG (Dako) and subsequently with rabbit peroxidase-antiperoxidase (Dako).

The immunohistochemical analysis was done in a blind fashion by two observers. Positive cells were counted in at least eight fields with a ×400 magnification and were expressed as number of positive cells/0.1 mm². P-selectin and ICAM expression was analyzed semiquantitatively in three different cortical regions: glomerulus, peritubular, perivascular, and intravascular. A scale was made ranging from 0 to 4: 0 (none), 1 (very mild), 2 (mild), 3 (moderate), and 4 (dense).

**Statistics**

For statistical analysis of ordinal data (macroscopy, histopathology, and adhesion-molecule expression), Krus
RESULTS

Macroscopical appearance

In both the ischemic and nonischemic allografted groups, the ureters had a normal caliber, with no signs of obstruction or leakage throughout the eight days of follow-up. Until day 4, the ischemic kidney allografts had a normal appearance. Six days after transplantation, however, five out of seven rats had a swollen kidney, with diffuse hemorrhages in the parenchyma. The renal artery was open in all cases; the ureter had a normal caliber. In nonischemic allografts, only one out of six kidneys showed the same changes at day 6 ($P = 0.06$). Two days later, no differences between both groups could be observed: All kidneys in both groups were rejected. The ureter and kidney from both ischemic and nonischemic isografts had a normal appearance at all time points studied.

Histology

The results analyzed according to the BANFF criteria are depicted in Figure 1. The histologic course in the allografts was as follows: At first, infiltration of the interstitium was observed, and thereafter, tubulitis, glomerulitis, and vasculitis developed. Exposure of the donor kidney to 24 hours of cold ischemia led to an earlier onset of the interstitial cell infiltration and tubulitis (Figs. 1 and 2). At day 2, some ischemic allografts showed infiltrating cells and tubulitis, and at day 3, all of these grafts had grade 1 interstitial cell infiltration, which was significantly higher than that in nonischemic allografts. Four days after engraftment, the degree of tubulitis was significantly higher in the ischemic allografts. From day 6, the interstitium of ischemic as well as nonischemic allografts was completely infiltrated by host mononuclear cells. By day 6, glomeruli and vessels were becoming involved in the process, leading to a full-blown acute rejection at day 8 with hemorrhagic infarctions in at least 70% of the kidney parenchyma, sometimes with thrombi in the midsize intraparenchymal arteries. Interestingly, two out of seven ischemic allografts had beginning fibrosis in the interstitium, which was not seen in the nonischemic group.

Nonischemic isografts showed no histologic changes during this period, whereas ischemic isografts showed some endothelial and tubular damage at days 2, 3, and 4. The endothelium was edematous, and some tubules had protein casts and lysosomal enzymes, observations that did not change the BANFF score in the ischemic isografts compared with the nonischemic ones. From day 6 and onward, no difference could be observed between ischemic and nonischemic isografts.
Fig. 2. (A) Normal tubulointerstitium of a kidney isograft at day 3 post-transplantation. (B) Lysosomal enzymes in tubulus cells of a 24-hour preserved kidney isograft at day 3. (C) Mild tubulitis and cellular infiltration in a nonischemic kidney allograft at day 3. (D) Moderate to severe tubulitis and cellular infiltration in a 24-hour preserved kidney allograft at day 3. (E) A normal intraparenchymal artery in a nonischemic kidney allograft at day 4. (F) Endothelial swelling and perivascular infiltration in a 24-hour preserved kidney allograft at day 4.

Immunohistology

In the nonischemic allografts, expression of the adhesion molecule P-selectin increased progressively in the peritubular capillaries during the first six days. By day 6, the glomeruli and the endothelium of intraparenchymal arteries also expressed P-selectin. Twenty-four hours of cold ischemia increased P-selectin expression, although this did not reach significance in the allogeneic setting. In addition, in ischemic kidneys, the expression of ICAM-1 was up-regulated by day 2. Thereafter, in the allografts, ICAM expression increased progressively, but the intensity on ischemic and nonischemic grafts was comparable.
Fig. 3. Cell infiltration in ischemic kidney isografts and allografts. In allografts, subjection of the kidney to 24 hours of cold ischemia led to an increased cell infiltration compared with nonischemic controls (*P < 0.05). In the first three days, the increase of CD45+ leukocytes consisted mainly of MHC class II+, CD4+ cells, and ED1+ macrophages. The peak of CD4+ cells was one day earlier, and the number of cytotoxic CD8+ T cells was higher at day 4. By day 6, the difference in cell infiltration between both groups had disappeared. Cell infiltration into ischemic isografts was also increased from day 3 and onward compared with nonischemic isografts, except for CD8+ T-cells (#P < 0.05). The increased infiltration of MHC class II+ and CD4+ cells after ischemia was significantly higher in allografts than in isografts at days 2 and 3 post-transplantation when compared with their nonischemic counterparts (*P < 0.05). Symbols are: (h, solid line) allograft; (j, solid line) allograft and 24 hours ischemia; (s, dotted line) isograft; (d, isograft 24 hours after ischemia).

From day 2 and onward, ischemia also induced expression of MHC class II antigens on the peritubular endothelium and increased its expression on the proximal tubular cells, in isografts and allografts to a similar degree. Concurrently with the up-regulated ICAM-1 and MHC class II expression on graft tissue, significantly higher numbers of CD45+ leukocytes infiltrated the ischemic allografts at days 2 and 3 (Fig. 3). Many of these leukocytes were MHC class II+ cells, CD4+ cells, and ED1+ macrophages (Fig. 3). Although the number of MHC class II+ cells did not change after day 4, the ratio of CD4- and CD8-positive cells was quite different between ischemic and nonischemic allografts. While the ischemic grafts had a maximal infiltration of CD4+ cells at day 3, with a rapid decline thereafter, the nonischemic allografts showed the highest number at day 4. While the number of CD4+ cells declined, the number of infiltrating CD8+ T lymphocytes progressively increased. ED1+ macrophages infiltrated the ischemic graft in higher numbers, but after day 3, the difference in number between ischemic and nonischemic allografts disappeared. NK cells infiltrated the grafts at the late stage of acute rejection. By day 6, 23 ± 17 NK cells/0.1 mm² were present in ischemic kidneys versus 15 ± 5 in nonischemic kidneys (P = NS).

In isografts, exposure to prolonged ischemia also led to a higher influx of CD45+ leukocytes, mostly consisting of CD4+ cells and ED1+ macrophages than in nonischemic isografts. Nonetheless, at days 2 and 3, the difference in number of CD45+, MHC class II+, and CD4+ cells between ischemic and nonischemic allografts was significantly greater than between both groups in the syngeneic setting.

DISCUSSION

In the present study, we demonstrate that prolonged cold ischemia results in an earlier onset of the histologic and immunologic features of rejection in kidney allografts. The mechanism through which ischemia increases the allogeneic immune response is not fully understood. While in isografts, 24 hours of ischemia led to an increase of the early adhesion molecule P-selectin at day 1 and ICAM-1 at day 2 on peritubular capillaries, in the allografts, ischemia did not affect their expression further. The expression of these adhesion molecules was persistently increased in both ischemic and nonischemic allo-
grafts. Increased P-selectin and ICAM-1 expression could be due to proinflammatory cytokines released by ischemia activated endothelium. This may mediate the selective migration of cell populations across endothelial barriers of the graft. Cytokines [interleukin-1, interferon-γ (IFN-γ)] of recruited and activated CD4+ cells, the numbers of which dramatically increased after day 1 in allografts, could be responsible for the ongoing endothelial cell expression of adhesion molecules in allografts.

In our study, both ischemic allografts and isografts had an induced expression of MHC class II on vascular endothelium and increased expression of MHC class II on the proximal tubular cells from day 2 and onward. Shoskes, Parfrey, and Halloran also demonstrated that transient ischemia in a nontransplant kidney led to an early up-regulation of MHC classes I and II mRNA transcripts and protein expression on tubular cells [8]. This observed MHC class II hyperexpression—triggered by the release of IFN-γ and other mediators [14, 15]—led to the hypothesis that ischemia could increase the immunogenicity of an organ graft. Recently, this suggestion has been fortified in an experimental lung allograft model: Lung allografts with prolonged cold ischemia had an increased expression of MHC class II antigens on the vascular endothelium and bronchial epithelium and a more severe rejection compared with allografts with a short ischemic period [9]. Since ischemic lung isografts were not included in this study, the higher degree of histologic rejection in ischemic lung allografts cannot be exclusively attributed to increased alloimmunogenicity by ischemia. Ischemia-induced histologic damage could be superimposed on allologeneic-mediated damage. Our data indicate that ischemia augments the alloimmune response since the increase of infiltration of MHC class II+ and CD4+ cells was significantly higher in allografts than in isografts at days 2 and 3 post-transplantation when compared with their nonischemic counterparts. In addition, in ischemic allografts, interstitial infiltrates and tubulitis developed earlier in the process of histologic rejection, whereas ischemia in isografts led to only minimal histologic changes including edematous swelling and tubules with protein casts and lysosomal enzymes. Interestingly, the increased CD4+ T-cell infiltration at days 2 and 3 in allografts did not correlate with the degree of MHC class II antigen expression on graft tissue, since allografts had a similar expression as isografts. Moreover, in contrast to the MHC class II antigen expression on endothelial cells that, together with costimulatory molecules, activate CD4+ T cells [16], the relevance of MHC class II hyperexpression on tubular epithelium is less clear. Epithelial cells do not seem to activate CD4+ cells because they miss costimulatory molecules, such as B7 [17, 18]. Nevertheless, even in a nonallologeneic setting, ischemia appears to activate CD4+ T cells. We recently demonstrated that cyclosporine therapy in ischemic rat kidney isografts inhibits early and late graft dysfunction [10]. Similarly, Takada et al found that blocking the CD28-B7 costimulatory pathway of T cells inhibits early and late ischemic kidney injury effectively [19].

In conclusion, ischemia augments allologeneic-mediated cell infiltration, the relevance of which in terms of cytokine expression is currently under investigation. The earlier onset of acute rejection in these allografts may be prevented by better preservation. Since recent evidence suggests that treatment of early histologic rejection in the absence of functional changes may improve the outcome after renal transplantation [20], tailored immunosuppression for allografts with a long cold ischemic time also may be beneficial.


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