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Endothelial nitric oxide synthase expression in ischemia-reperfusion injury after living related-donor renal transplantation

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Abstract Ischemia-reperfusion injury during renal transplantation has been linked to early graft dysfunction and late graft failure. Nitric oxide (NO), produced by NO synthase (NOS), participates in the recovery from ischemia. We correlated the intensity of graft immunoreactivity for the endothelial NOS isoform (eNOS) during early reperfusion with graft function in 25 children receiving grafts from related donors. Renal allograft biopsy specimens were obtained before transplantation, 1 h after renal artery reperfusion, and 1 year after transplantation. Immunohistochemical staining for eNOS occurred mainly within the endothelium of glomerular capillaries and peritubular capillaries as well as in tubule cells. The mean intensity score for eNOS staining (0–9) was 3.0 ± 1.4 before transplantation, 4.5 ± 1.9 at 1 h, and 3.3 ± 1.9 at 1 year (baseline vs 1 h, $P < 0.05$). Creatinine clearance (ml/min) in patients with a 1-h eNOS score of below 5 and of at least 5, respectively, was 77.1 ± 28.4 vs 104.3 ± 25.3 at 1 month, 78.7 ± 33.4 vs 105.2 ± 24.4 at 3 months, 64.7 ± 30.1 vs 100.1 ± 25.3 at 1 year, 58.2 ± 31.3 vs 84.7 ± 18.8 at 3 years, and 71.2 ± 19.7 vs 78.3 ± 23.1 at 5 years ($P < 0.05$ for 1 month, 1 year, and 3 years). We concluded that elevated eNOS expression after reperfusion in living related-donor renal transplantation enhances the recovery from renal ischemia and, consequently, reduces late graft deterioration.

Keywords Renal transplantation · Living related donors Ischemia-reperfusion injury · Endothelial nitric oxide synthase · Acute rejection · Chronic allograft nephropathy

Ischemia-reperfusion (I-R) injury in cadaveric renal transplantation may cause acute tubular necrosis or delayed initiation of graft function [30]. I-R injury was correlated with the incidence of acute rejection in several clinical series [8, 12, 21]. Experimental and clinical evidence has also identified I-R injury as an antigen-independent risk factor for chronic renal allograft failure [12, 13, 29, 35].

Nitric oxide (NO) appears to be a key link between I-R injury and the rate of tissue repair during injury response, the number of acute rejection episodes, and the occurrence of chronic allograft nephropathy [14]. A versatile intercellular messenger molecule associated with vasodilation and neurotransmission, NO is also involved in inflammation, tissue injury, and cell defense [19, 28]. Synthesis of NO is catalyzed by nitric oxygen synthase (NOS) [19], which has three known isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) [22, 23, 28]. The isoform predominantly involved in the recovery from I-R injury in the transplanted kidney is eNOS [28, 32]. The activity of eNOS after I-R can thus influence the

degree of ischemic damage and the rate of recovery from injury.

In this study we immunohistochemically investigated patterns of eNOS expression in renal grafts from living related donors to examine the significance of eNOS expression in biopsy specimens obtained 1 h after reperfusion for subsequent graft function.

Patients and methods

Patients

Between 1986 and 1999, a total of 55 renal grafts from living related donors was transplanted in 55 recipients at Kobe University Hospital. Immunosuppressive therapy included intravenous infusion of cyclosporin A (CyA) for 3 or 4 days, followed by sufficient oral CyA to achieve a trough concentration of 200–250 ng/ml during the 1st month. Trough CyA concentrations during the 2nd and 3rd month were set at 150–200 ng/ml and 100–150 ng/ml, respectively. CyA concentrations were measured in whole blood shortly before the next dose. The mean CyA trough concentration was calculated from CyA levels at days 1, 7, 14, 30, 60, 90, and at 1 year. The induction regimen given in addition to CyA included mizoribine (2–4 mg/kg), methylprednisolone (1 mg/kg per day), and antilymphocyte globulin or deoxyspergualin (3 mg/kg per day).

Allograft biopsies were performed three times for each patient. Immediately before transplantation and at 1 h after reperfusion, cortical-wedge biopsy of the transplanted kidney was performed. At approximately 1 year after transplantation, a core-needle biopsy was performed with a Biopty gun (18G; C.R. Bard, Covington, Ga. USA) under ultrasonographic guidance with the informed consent of all patients or parents, depending on patient age. In this study we excluded the cases in which recurrent nephropathy was encountered and which lacked the biopsy. Finally, 25 patients were examined in this study. Biopsy specimens were assessed according to the Banff working classification by two observers in a blinded fashion. Acute rejection was diagnosed by an increase in serum creatinine concentration exceeding 30%; whenever possible, a core biopsy specimen of the graft was obtained to confirm the diagnosis.

Immunohistochemistry

Paraffin-embedded sections 2-μ m in thickness were cut from pretransplantation specimens ($n=25$), 1-h specimens ($n=25$), and 1-year specimens ($n=23$) for eNOS immunostaining by an indirect immunoperoxidase method using an avidin-biotin-peroxidase kit (Vector Laboratories, Burlingame, Calif., USA) and mouse monoclonal antibody against human eNOS (Transduction Laboratories, Lexington, Ky., USA). After the paraffin had been removed with xylene, the tissue sections were rehydrated in graded ethanol solutions and washed in phosphate-buffered saline (PBS). Endogenous peroxidase was inactivated by incubation for 30 min at 37 °C in a methanol/peroxide solution (0.03%). After non-specific binding had been blocked with 1.5% normal horse serum in 0.5% PBS, sections were incubated overnight at 4 °C with primary antibody. Bound antibody was localized with biotinylated horse anti-mouse IgG and avidin-peroxidase complex. The reaction product was stained with 3,3′-diaminobenzidine (Sigma Chemical, St. Louis, Mo., USA). The sections were counterstained with 1% methyl green.

Immunoreactivity was assessed semiquantitatively. In blinded fashion, two observers applied a 9-point scoring system, taking into account the extent of staining in peritubular capillaries, glomeruli, and tubules (Table 1). In brief, the score of each component (peritubular capillaries, glomeruli, tubules), which was determined by the percentage of stained area as shown in Table 1, were summed for each sample. To investigate the effect of eNOS expression at 1 h after reperfusion on subsequent clinical outcome, we used the results of immunohistochemical staining at 1 h to assign cases to one of two groups: group 1, low eNOS expression (total score 0–4); or group 2, high eNOS expression (total score 5–9). Creatinine clearance (Ccr) of groups 1 and 2 was compared at multiple time points. Staining scores in grafts were compared for pre-transplantation, 1 h, and 1 year after transplantation.

Table 1 Semi-quantitative scoring of immunostaining. Staining intensities in tubule cells, endothelial cells of glomerular capillaries and epithelium of Bowman's capsule, and endothelium of the capillaries in the tubule interstitium, were each scored separately from 0 to 3 and summed as the eNOS staining score for the specimen (0–9)

Score	No staining (0%)	Mild staining (1%–25%)	Moderate staining (26%–50%)	Strong staining (>50%)
Tubule cells	0	1	2	3
Glomerular	0	1	2	3
Interstitial	0	1	2	3

Statistical analysis

The Mann-Whitney U test was used to compare Ccr of groups 1 and 2 and to compare eNOS-staining scores of graft specimens obtained at 1 h after transplantation with those obtained before or 1 year after transplantation.

Results

Immunohistochemical staining

Immunoreactivity was demonstrated by anti-eNOS antibody in the endothelium of peritubular capillaries and in cells of proximal tubules and glomeruli. Staining in glomeruli was seen mainly in glomerular capillaries, but was also seen occasionally in the epithelium of Bowman's capsule (Fig. 1).

Expression of eNOS in grafts over time

The mean eNOS expression score before transplantation was 3.0 ± 1.4. This rose to 4.5 ± 1.9 ($P<0.05$ vs pretransplantation) at 1 h after reperfusion and returned to near baseline at 1 year (3.3 ± 1.9, $P<0.05$ vs 1 h after reperfusion; Fig. 2).

eNOS expression, clinical parameters, and graft function

Patient age at transplantation was 12.3 ± 5.1 years (Table 2). The mean follow-up period was 3.8 years. Patients were classified into two groups according to staining score as mentioned above. The mean number of

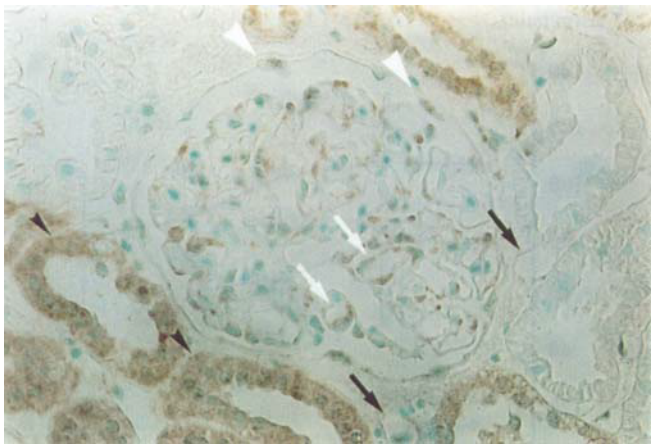

Fig. 1 Localization of immunostaining for eNOS in an allograft at 1 h after reperfusion. Immunoreactivity for eNOS was seen in the endothelium of peritubular capillaries (*black arrow*) and in cells of proximal tubules (*black arrowhead*) and glomeruli. Glomerular staining was limited to the epithelium of Bowman's capsule (*white arrow*) and glomerular capillaries (*white arrowhead*); methyl green, magnification ×40

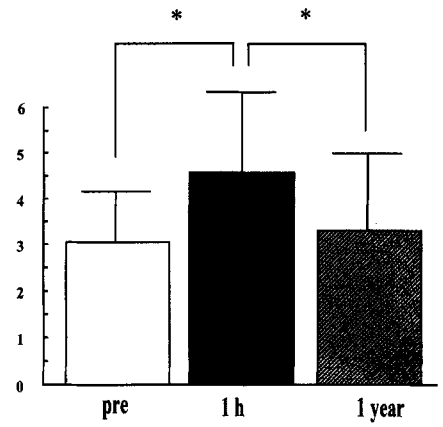

Fig. 2 Mean eNOS staining scores in specimens from pre-transplantation grafts (*pre*), specimens from allografts at 1 h after reperfusion, and allograft specimens at 1 year after transplantation were 3.0 ± 1.4, 4.5 ± 1.9, and 3.3 ± 1.9, respectively. Expression of eNOS was significantly increased at 1 h after reperfusion and later declined to near-baseline values at 1 year. $*P < 0.05$ for pre vs 1 h and 1 h vs 1 year

acute rejection episodes in group 1 (low expression, score 0–4) and 2 (high expression, score 5–9) was similar: 0.8 ± 1.1 and 0.92 ± 1.4, respectively. Ccr (ml/min) in groups 1 and 2, respectively, was 77.1 ± 28.4 ($n = 12$) vs 104.3 ± 25.3 ($n = 13$) at 1 month, 78.7 ± 33.43 ($n = 12$) vs 105.2 ± 24.4 ($n = 13$) at 3 months, 64.7 ± 30.1 ($n = 12$) vs 100.1 ± 25.3 ($n = 13$) at 1 year, 58.2 ± 31.3 ($n = 10$) vs 84.7 ± 18.8 ($n = 13$) at 3 years, and 71.2 ± 19.7 ($n = 5$) vs 78.3 ± 23.1 ($n = 11$) at 5 years (Fig. 3). Ccr was significantly higher in group 2 than in group 1at 1 month, 1 year, and 3 years ($P < 0.05$). No significant differences were detected between groups 1 and 2 concerning ischemic time, CyA trough level, and episodes of rejection (Table 2).

Discussion

I-R injury occurring secondarily to kidney retrieval, storage, and transplantation, affects the early phase of recovery following kidney transplantation [2, 30] and has also been identified as an antigen-independent risk factor for chronic renal allograft failure [8, 12, 21, 33]. How I-R injury influences long-term allograft function is unclear, but recent studies suggest that allografts exposed to I-R injury have increased immunogenicity, leading to increased acute rejection, which is a known risk factor for chronic renal damage [31]. In addition, I-R injury causes a release of cytokines and growth factors associated with chronic allograft nephropathy [20]. NO, which has a proven role in renal vasodilation, tubuloglomerular feedback, sodium excretion, and angiotensin regulation in the normal kidney [3], exerts several beneficial effects on the process of recovery from I-R injury to the kidney. Inhibition by NO of platelet adhesion and aggregation decreases the occurrence of vascular thrombosis during reperfusion [10, 26]. NO also interferes with monocyte adherence and migration [4, 9] as well as leukocyte activation, which leads to neutrophil-endothelium adhesion [15, 27] and the generation of oxygen free radicals. Moreover, NO induces relaxation of pre-glomerular arteries to improve renal blood flow and oxygenation [5] and is reported to have an overall protective effect on tissues exposed to I-R injury [11, 36]. Therefore, NO is a potential key molecule in the link between I-R injury and tissue repair.

Under physiological conditions, renal NO is derived mainly from constitutive NOS, including eNOS and nNOS [25, 28]. Shoskes et al. found a significant increase in eNOS activity in the ischemic kidney for the first 6 h after reperfusion [32]. We therefore examined eNOS expression after reperfusion in renal transplants from living related donors. Moreover, expression in biopsy specimens obtained 1 h after reperfusion was investigated in terms of the associated number of subsequent acute rejection episodes and both early and late graft function after transplantation. We found that compared with pre-transplantation specimens, eNOS expression in grafts was significantly increased at 1 h after reperfusion, but had returned to normal in allografts studied 1 year after transplantation. eNOS is a Ca^{2+}-dependent constitutive enzyme, and the elevated eNOS expression at 2 h after reperfusion can be attributed to vascular shear stress during reperfusion as well as intracellular calcium accumulation resulting from ischemia [1, 34]. A bi-phasic response of eNOS activity to I-R injury in the rat kidney has also been reported, with early stimulation

Table 2 Patient characteristics. Patient characteristics did not differ significantly between groups 1 and 2. CyA trough levels (mean) were calculated from CyA levels at days 1, 7, 14, 30, 60, 90, and at 1 year (*TIT* total ischemia time, *CIT* cold ischemia time, *FSGS* focal segmental glomerulosclerosis, *IgA* IgA nephropathy, *Alport* Alport syndrome, *MPGN* membranoproliferative glomerulonephritis, *Hypo* congenital hypoplastic kidney, *HUS* hemolytic uremic syndrome, *CGN* chronic glomerulonephritis, *CNS* congenital nephrotic syndrome, *RN* rheumatoid nephropathy)

Characteristic	Group 1 ($n=12$)	Group 2 ($n=13$)
Age (years)	13.2 ± 5.6	11.5 ± 4.8
Gender (M:F)	8:4	9:4
Body weight (kg)	28.3 ± 11.2	29.7 ± 13.8
Height (cm)	123.8 ± 36.6	128.2 ± 35.4
HLA mismatch		
A, B	1.89 ± 0.33	1.64 ± 0.67
DR	0.67 ± 0.50	0.91 ± 0.30
CyA trough level		
1 day after surgery	244.1 ± 177.2	199.4 ± 143.5
Mean	175.7 ± 87.3	188.1 ± 27.7
Donor age (years)	41.1 ± 5.5	43.2 ± 7.5
M:F	5:7	3:10
TIT (min)	62.1 ± 15.7	65.4 ± 26.3
CIT (min)	58.7 ± 15.0	61.4 ± 25.9
Rejection	0.8 ± 1.1	0.9 ± 1.4
Primary renal disease		
FSGS:IgA:Alport:MPGN: Hypo:HUS:CGN:CNS:RN	4:2:1:1:0:1:1:1:1	9:1:2:0:1:0:0:0:0

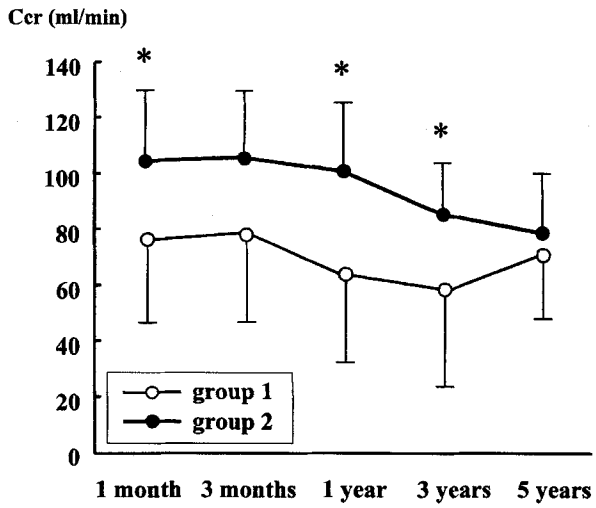

Fig. 3 Change in Ccr in patients of groups 1 and 2 at 1 month, 3 months, 1 year, 3 years, and 5 years aftser transplantation (mean ± SD). *$P < 0.05$, group 1 vs 2

followed by a period of depressed activity. This change of activity occurred despite the presence of a persistently augmented content of eNOS protein. Although the expression of eNOS protein is not always consistent with its activity throughout the course, the ischemic kidney showed the highest expression of eNOS protein and the peak level of eNOS activity at 2 h after reperfusion [32]. Although this NOS activity is not always in correlation with a normal level of NO, reduced NOS activity could be deleterious to the recovery from ischemia due to the resultant intra-renal vasoconstriction [38]. Chintala et al. [7] also reported that inhibition of eNOS activity caused excessive vasoconstriction and exacerbated organ ischemia, microvascular thrombosis, and mortality. Therefore, the measurement of eNOS expression in our study was critical to investigate the effect of NO on I-R injury.

The cadaveric donor kidney subjected to varying degrees of warm and cold ischemia may be damaged by a prolonged lack of oxygen and energy-producing substrates, or more commonly, by reactive oxygen species generated after reperfusion. I-R injury in cadaveric renal transplants causes the histological picture of acute tubular necrosis (ATN) and often results in clinically evident delayed graft function (DGF) [30]. In our living related-donor transplants, little warm ischemia occurred, and the duration of cold ischemia was limited. Biopsy specimens from 1 h after reperfusion showed no evidence of ATN, and no patients manifested DGF. Thus, in the absence of clinical or histopathological sequelae of ischemia, the I-R insult induces eNOS expression that may support recovery from I-R in living related-donor transplantation. In addition, renal function after transplantation was compared for up to 5 years for patients whose grafts showed high eNOS expression at 1 h (group 2) and for those with low expression (group 1). Ccr in group 2 was consistently higher than in group 1 in follow-up evaluations. Although the number of acute rejection episodes did not differ between the two groups, long-term graft function was consistently better in the group with high eNOS expression. These results suggest that NO synthesized by eNOS might reduce damage from I-R injury and improve recovery, resulting in better graft function.

In this study we did not examine inducible NOS (iNOS), which is constitutively expressed in several segments of the renal tubule as well as in the glomerulus and interlobular and arcuate arteries of the normal rat kidney [18]. However, high levels of NO produced by iNOS have been implicated in the renal dysfunction/injury associated with renal ischemic reperfusion [37]. Several in-vivo and in-vitro investigations have demonstrated that inhibition of iNOS expression and iNOS activity can prevent NO-mediated renal injury [16, 17, 24, 36, 37]. Thus, both eNOS and iNOS expression can occur simultaneously after reperfusion, and we believe that the beneficial local effect of eNOS activity on the renal vasculature would not be offset by the detrimental cytotoxic effects of iNOS from infiltrating macrophages.

Calo et al. reported a CyA-induced NO system up-regulation in transplanted patients. Endothelial NO is crucial in the maintenance of a state of basal dilation, and recent studies have suggested an NO-mediated counter-regulatory mechanism to be protective from CyA-induced vasoconstriction [6]. In addition, the NO-mediated counter-regulatory system to CyA-induced vasoconstriction could be deleted in patients by

CyA-induced superoxide and free radical production, which, by increasing NO metabolism, could contribute to CyA-induced vasoconstriction. In our study we could not find any difference in CyA levels at 1 day after transplantation between patients with high expression of eNOS and those with low expression.

To our knowledge, our study is the first to report elevated eNOS expression after reperfusion in human kidneys from living related donors. High expression enhanced early recovery as well as late graft function.

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