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

Takeshi Ishimura
Masato Fujisawa
Shuji Isotani
Kazumoto Iijima
Norishige Yoshikawa
Sadao Kamidono

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

Introduction

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 anti-lymphocyte 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 Biopby gu (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-μm in thickness were cut from pre-transplantation 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.

#### Statistical analysis

The Mann-Whitney U test was used to compare Ccr of groups 1 and 2 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 pre-transplantation) 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).

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)

<table>
<thead>
<tr>
<th>Score</th>
<th>No staining (0%)</th>
<th>Mild staining (1%–25%)</th>
<th>Moderate staining (26%–50%)</th>
<th>Strong staining (&gt; 50%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubule cells</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Glomerular</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Interstitial</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

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