Oxidative Stress in Kidney Transplant Biopsies

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Abstract

Objectives: Kidney allograft biopsies are performed after kidney transplant to determine graft dysfunction. We aimed to define and measure the oxidative stress occurring in these biopsies and compared these biopsies with donor pretransplant biopsies.

Materials and Methods: The biopsy procedure was done according to the unit protocol. A core of tissue was taken for research purposes only when it was safe enough to proceed for an extra core. Common indications for biopsy were acute or chronic graft dysfunction, delayed graft function, acute cellular rejection, and calcineurin toxicity. There were 17 pretransplant biopsies taken from deceased-donor kidneys. Biopsy specimens were snap frozen immediately in liquid nitrogen and stored at -70°C. Samples were processed for Western blot and tested for markers of oxidative stress.

Results: There were 61 biopsies analyzed. Oxidative stress enzymes were evaluated by Western blot including catalase, manganese superoxide dismutase, copper zinc superoxide dismutase, thioredoxin reductase, and thioredoxin. Up-regulation of most antioxidant enzymes was observed in pretransplant biopsies. Increased expression of manganese superoxide dismutase was observed in donor kidneys and kidneys with acute cellular rejection and calcineurin toxicity. Copper zinc superoxide dismutase and catalase were elevated in donor and acute cellular rejection biopsies. Thioredoxin was elevated in donor biopsies and thioredoxin reductases were elevated in donor biopsies and biopsies with acute cellular rejection and calcineurin toxicity.

Conclusions: The kidney allograft biopsies showed that oxidative stress levels were elevated during allograft dysfunction in all biopsies regardless of diagnosis, but not significantly. The levels also were elevated in pretransplant biopsies. The study showed that oxidative stress is involved in various acute injuries occurring within the allograft.

Key words: Catalase, Copper zinc superoxide dismutase, Manganese superoxide dismutase, Oxidation, Thioredoxin, Thioredoxin reductase

Introduction

Kidney transplant offers patients with end-stage renal failure improved survival and quality of life compared with dialysis. Overall long-term graft and patient survival have remained unchanged since 1995. This lack of improvement has been observed despite reduced early and late acute rejection rates1 resulting in 5-year graft survival of 70% and 10-year graft survival of 50%.2

“Oxidative stress” was a term first described in 1985 as a disturbance in the pro-oxidant-antioxidant balance in favor of the former.3 However, it is important that reactions involving free radicals are not necessarily deleterious; on the contrary, they are of fundamental importance for life because free radicals take part in key biochemical reactions in all living organisms. A more useful contemporary definition is a disruption of redox signaling and control.4 Reactive oxygen intermediates can affect the signaling of a wide variety of kinase pathways such as the mitogen-activated protein kinases/extracellular...
signal-regulated kinases and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathways. These kinase pathways can have both antiapoptotic and apoptotic roles. Reactive oxygen species can participate in neutrophil recruitment by up-regulation of adhesion molecules and chemotactic factors. Oxidative stress also is increased in patients with chronic allograft nephropathy (CAN). We aimed to define and measure the oxidative stress occurring in kidney allograft biopsies and to compare these biopsies with donor pretransplant biopsies.

Materials and Methods

Biopsies

Kidney transplant graft biopsies were collected from patients who were undergoing the biopsy procedure because of graft dysfunction. The common indications for biopsy were delayed graft function (DGF), acute cellular rejection (ACR), calcineurin toxicity, and CAN. The procedure was done under local anesthesia according to the unit protocol with ultrasonography guidance. An automatic biopsy gun was used with a 16-F Tru-Cut needle. There was 1 core of tissue taken for research purposes only when it was safe to proceed for an extra core and the patient was comfortable and gave verbal consent. Some biopsy samples were obtained from deceased-donor kidneys before transplant only when the family gave consent for the organ to be used for research purposes. The biopsy specimens were snap frozen immediately in liquid nitrogen and stored at -70°C until further analysis. Samples were later processed for Western blot and tested for markers of oxidative stress. Biopsies were taken from the patients undergoing kidney transplant biopsies for any clinical indication after signing an informed consent form.

Informed written consent was obtained from all patients. Ethical approval was granted by the Liverpool adult local research ethics committee before the study. All of the protocols conformed with the ethical guidelines of the 1975 Helsinki Declaration.

Analyses

Biopsy specimens were taken from the -70°C freezer and ground mechanically in liquid nitrogen. Homogenization buffer was added and the mixture was centrifuged at 16000 × g at 4°C for 10 minutes; the supernatants were stored at -70°C. The protein concentration of the sample was determined using the bicinchoninic acid assay. Aliquots of samples containing 40 μg total protein were heated for 5 minutes at 95°C in denaturing loading buffer. The proteins were resolved on 4% and 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis mini gels and blotted onto nitrocellulose membranes. The membranes were blocked for 1 hour at room temperature in 5% skim milk in phosphate-buffered saline (PBS) (Blotto, Santa Cruz Biotechnology, Dallas, TX, USA) supplemented with polysorbate surfactant (0.05% Tween 20, Promega Corporation, Madison, WI, USA) and exposed at room temperature to primary antibodies using antibody dilutions and times recommended by the manufacturer. The membranes were repeatedly washed in PBST and exposed at room temperature to secondary antibodies as recommended by the manufacturer. Protein bands were visualized using chemiluminescent substrate (Super Signal West Dura Extended Duration, Pierce Perbio Science, Rockford, IL, USA) and recorded using an imaging system and software (Chemidoc XRS and Quantity One, Bio-Rad Laboratories, Hercules, CA, USA). The intensity of the signal from each band was corrected for background signal and expressed as a percentage of the content of control cells. Western blot analysis was done on several gels because of the large number of samples. On each gel, samples were loaded from different clinical conditions. Beta-actin was used as loading control. Quantification graphs were produced as a percent change from a CAN sample from each individual gel by performing densitometry. Data were analyzed using software (Prism 5, GraphPad Software, San Diego, CA, USA).

Results

Biopsies

There were 61 kidney allograft biopsies collected from kidney transplant recipients who were undergoing this procedure for various clinical reasons; 47 of these biopsies were analyzed and their data were presented. There were 17 pretransplant biopsies from deceased-donor kidneys that were included in this study.

Demographic characteristics of kidney transplant recipients

There were 20 females (42.6%) and 27 males (57.4%). Median age of the recipients was 44.6 ± 2.13 years (range, 19.4-76.9 y). There were 35 deceased-donor
kidney transplants (74.5%) and 12 living-donor kidney transplants (25.5%). There were 11 patients who had a first kidney transplant from a living donor, and 1 patient who had a third kidney transplant from a living donor; there were 29 patients who had their first kidney transplant from a deceased donor, 5 patients who had a second kidney transplant from a deceased donor, and 1 patient had a third kidney transplant from a deceased donor.

Clinical indications for biopsy
The most common indication for undergoing kidney biopsy was renal dysfunction which was usually indicated by a rise in serum creatinine ≥ 10%. There were 34 patients (72.3%) who had renal dysfunction, 8 patients (17%) who had DGF, 4 patients (8.5%) who had renal dysfunction associated with proteinuria, and 1 patient who underwent biopsy because of proteinuria without associated renal dysfunction. Median time between the date of transplant and biopsy was 157 ± 275.7 days (range, 6-8878 d).

Histologic diagnosis was available for all 47 allograft biopsies. Diagnosis was CAN in 14 biopsies (29.8%), acute tubular necrosis (ATN) in 5 biopsies (10.6%), ACR in 19 biopsies (40.4%) (ACR: borderline, 7 biopsies; grade 1A, 9 biopsies; grade 2A, 3 biopsies). Cyclosporine toxicity was observed in 4 biopsies (8.5%) and other diagnoses were noted (Table 1). Banff 97 classification 8 was used to score the grade of ACR and chronic changes in all allograft biopsies.

### Table 1. Histologic Diagnosis of Kidney Allograft Biopsies

<table>
<thead>
<tr>
<th>Diagnosis on Biopsy</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute tubular necrosis</td>
<td>5</td>
</tr>
<tr>
<td>Borderline rejection</td>
<td>7</td>
</tr>
<tr>
<td>Chronic allograft nephropathy</td>
<td>14</td>
</tr>
<tr>
<td>Cyclosporine toxicity</td>
<td>4</td>
</tr>
<tr>
<td>Acute rejection (grade 1A)</td>
<td>9</td>
</tr>
<tr>
<td>Acute rejection (grade 2A)</td>
<td>3</td>
</tr>
<tr>
<td>Hemolytic uremic syndrome</td>
<td>1</td>
</tr>
<tr>
<td>Immunoglobulin A nephropathy</td>
<td>2</td>
</tr>
<tr>
<td>Normal</td>
<td>1</td>
</tr>
<tr>
<td>Posttransplant lymphoproliferative disease</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
</tr>
</tbody>
</table>

Analysis of graft biopsies for oxidative stress
There were 109 biopsy samples analyzed. The following antioxidant proteins were detected: catalase (60 kilodaltons), manganese superoxide dismutase (MnSoD) (25 kilodaltons), copper zinc superoxide dismutase (CuZnSoD) (23 kilodaltons), thioredoxin reductase (55 kilodaltons), thioredoxin (2b1) (12 kilodaltons), and thioredoxin (62 kilodaltons).

For quantification, each gel had 1 sample from a kidney with CAN that was used as a control because there was no adequate control biopsy (Figure 1). For quantification, 1 biopsy that was reported as normal, 1 biopsy with immunoglobulin A nephropathy, and 2 biopsies with CAN were grouped together and results were expressed as a percentage change compared with this group. There were 2 biopsies from the ACR and donor group. The CuZnSoD density was higher in biopsies taken from ACR and donor kidneys than CAN samples. However, only donor biopsy samples showed a significant increase (P < .05) (Figure 2). The MnSoD also was increased in both donors and ACR kidneys but did not reach statistical significance. Catalase and thioredoxin reductase were similar between ACR and donor biopsies (not significant). Thioredoxin reductase was elevated in ACR biopsies compared with donor biopsies.

![Figure 1. Representative Western Blot](image1)

**Abbreviations:** CAN, chronic allograft nephropathy; N, normal. Increased expression of manganese superoxide dismutase (MnSoD), copper zinc superoxide dismutase (CuZnSoD), and catalase in donor biopsies (D) and kidneys with acute cellular rejection (ACR).

![Figure 2. Quantification of Gel 1 Showing Copper Zinc Superoxide Dismutase (CuZnSoD), Manganese Superoxide Dismutase (MnSoD), Catalase, and Thioredoxin 55](image2)

**Abbreviations:** ACR, acute cellular rejection; CAN, chronic allograft nephropathy; D, donor (*P < .05*).
from the ATN group that also showed ACR; for quantification, this was pooled with the ATN results. Catalase was significantly higher in the donor group \((P \leq .05)\). The CuZnSoD also was significantly higher in the donor group than ACR or ATN groups but not higher than the CAN group. Thioredoxin reductase also was significantly higher in the donor group than ATN and ACR groups but not the CAN group, similar to the pattern observed with CuZnSoD. Donor MnSoD had a significant increase compared with all other groups \((P \leq .05)\) (Figure 3).

The results of all Western blots were combined and plotted together. The MnSoD, CuZnSoD, and thioredoxin reductase 55 were increased in deceased-donor, ACR, and cyclosporine toxicity groups but this did not reach statistical significance. All enzymes tested showed reduced expression in CAN biopsies (Figure 4).

**Discussion**

This experimental work on kidney allograft biopsies was aimed to determine the oxidative stress occurring after transplant. Donor pretransplant biopsies provided a unique opportunity to detect oxidative stress occurring because of hypoxia and cold storage. Kidney allograft biopsies are performed routinely to evaluate renal dysfunction. The procedure generally has a low complication rate. The following complications have been reported: gross hematuria, 3.5%; perirenal hematoma, 2.5%; arteriovenous fistula, 7.3%; and vasovagal reaction, 0.5%. Major complications requiring invasive procedures such as blood transfusions or urinary catheterization are observed in 1% cases. Some authors have advocated kidney biopsies for prognostic purposes. Morphologic changes of CAN expressed as tubular atrophy and interstitial fibrosis precede the decline of kidney function. Other authors have suggested the use of biopsies to detect subclinical rejection.

A total of 61 biopsies were analyzed and the following oxidative stress enzymes were detected by Western blot: catalase (60 kilodaltons), MnSoD (25 kilodaltons), CuZnSoD (23 kilodaltons), thioredoxin reductase (55 kilodaltons), thioredoxin (2b1) (12 kilodaltons), and thioredoxin (62 kilodaltons).

Up-regulation of most antioxidant enzymes was detected in biopsies obtained from deceased-donor kidneys before transplant. The MnSoD was expressed more in donor kidneys and kidneys with ACR and cyclosporine toxicity. The CuZnSoD was also elevated in donor and ACR biopsies. Catalase was elevated in donor and ACR biopsy samples. Thioredoxin was elevated in donor biopsies, and thioredoxin reductases were elevated in donor, ACR, and cyclosporine toxicity biopsies. Ischemic injury and related oxidative stress possibly are reasons for the elevation in these enzymes. However, because of lack of adequate control biopsies, the results were difficult to interpret.

Catalase has been investigated by several authors in biopsy tissue. In a proteome analysis of rat kidney allografts, the expression of catalase was down-regulated. Polymorphism in the catalase gene is associated with DGF in kidney allograft recipients. Antioxidative defense was higher in the tubulo-interstitial compartment than glomerular cells, and a reduction was observed in glomerular enzymes catalase and glutathione peroxidase 3 and 4 in nephritic kidneys; tubular gene expression was down-regulated for catalase, glutathione peroxidase 3, and thioredoxin reductase 1 and 2.
Compared with living-donor kidneys, deceased-donor kidneys have a distinctly different set of antioxidant genes originating from the tubulo-interstitial compartment. The deceased-donor kidneys have long periods of cold storage resulting in hypoxic damage and oxidative stress. Our results from such kidneys confirm this phenomenon because biopsies taken from these kidneys showed an increase in oxidative stress enzymes such as MnSoD, CuZnSoD, catalase, and thioredoxin enzymes. Donor kidneys from recipients with impaired allograft function also showed activation of genes mainly belonging to the functional classes of immunity, signal transduction, and oxidative stress response.

The CuZnSoD comprises 90% total superoxide dismutase activity in eukaryotic cells. An increase in CuZnSoD has been reported from cattle liver biopsies after inducing oxidative stress. Genetic expression of mitochondrial MnSoD, but not cytosolic CuZnSoD or glutathione peroxidase, increased with cold exposure, suggesting mitochondria as a cellular source of free radicals and activation under cold injury.

The MnSoD overexpression in renal tubular cells protects against high-glucose-induced oxidative stress. Increased mRNA levels of CuZnSoD and MnSoD enzymes have been observed in the cortex and medulla of rats subjected to hypoxia. In a study of the role of MnSoD and mitochondrial injury in cold preservation, short-term cold ischemia-reperfusion resulted in inactivation of MnSoD; this suggested that compounds designed to prevent early mitochondrial injury in kidneys that undergo cold preservation would improve kidney function and graft survival after transplant. Loss of mRNA of catalase and glutathione peroxidase may be the first markers of alterations in cellular redox status in ischemia-reperfusion injury. The mRNA for MnSoD was up-regulated at all times with ischemia-reperfusion injury, suggesting that antioxidant genes are not coordinately expressed during ischemia-reperfusion and that the differential loss of antioxidant enzymes may contribute to the heterogeneous kidney tissue damage as a result of ischemia-reperfusion induced oxidative stress.

Some authors also have suggested that mitochondrial dysfunction is an early event in a rat model of allotransplant and may cause the development of CAN.

Thioredoxin reductase is prominently expressed in the proximal tubules of rodent kidneys. Thioredoxin is secreted from proximal tubules into urine during renal ischemia-reperfusion and may have a protective effect against renal ischemia-reperfusion injury. Elevated thioredoxin reductase activity has been observed after exposure to lead acetate in rat kidneys along with other antioxidant enzymes such as catalase and superoxide dismutase. In proximal tubules, nuclear and luminal localization was detected for thioredoxin 1 after ischemia-reperfusion injury. The cytosolic thioredoxin R1 was detected in all tubular segments, especially strongly in distal convoluted tubules and thin segments of the inner medulla. Thioredoxin 2 was diffusely expressed in all regions of the kidney. After ischemia-reperfusion injury, thioredoxin 2 immunoreactivity increased in the mTAL segments and in the lumen of thin segments in the inner medulla. Thioredoxin R2, which was detected primarily in connective tissue in the sham group, showed a segment-specific increase after ischemia-reperfusion injury, most notably in the lumina and epithelial cells of distal convoluted tubules. Strong immunoreactivity for thioredoxin R2 in luminal compartments also was observed in the inner medulla.

Several biopsy specimens studied were taken from patients with cyclosporine nephrotoxicity, and the histology was characterized by tubular vacuolation and an ATN-like appearance. Results from Western blot assay revealed that all antioxidant enzymes tested were elevated in cyclosporine nephrotoxicity. Thioredoxin reductase showed the maximum elevation but other enzymes were elevated including MnSoD, CuZnSoD, catalase, and thioredoxin (12 kilodaltons). Cyclosporine nephrotoxicity is manifested by renal insufficiency due to glomerular disease and abnormalities in tubular function. Several mechanisms have been proposed for cyclosporine-induced nephrotoxicity, such as sodium retention, renal vasoconstriction, renal hypoxia as a consequence of renal vasoconstriction, stimulation of the renin-angiotensin system, activation of the sympathetic nervous system, impaired synthesis of nitric oxide, and increased growth factor B1.

Oxidative stress has been implicated in cyclosporine nephrotoxicity. Increase in reactive oxygen species generation and lipid peroxidation may affect renal function and interstitial fibrosis.
A previous report showed reduction of both oxidative stress and increased inducible nitric oxide synthase (iNOS) and NF-kB expression induced by cyclosporine with use of red wine polyphenol. Treatment with N-acetyl cysteine significantly protected animals against cyclosporine-induced structural and functional impairment of kidneys, implicating the role of oxidative stress in the pathogenesis of cyclosporine-induced nephrotoxicity. Cyclosporine nephrotoxicity is mediated by increased expression of iNOS, NF-kB, and matrix metalloproteinase 2, which was altered by the antioxidant S-allylcysteine, rendering protection to the kidney cells. Histologic evaluation before transplant may enable the identification of organs unsuitable for single transplant, but such evaluation of oxidative stress enzymes is not performed in routine practice.

In summary, it is generally believed that reactive oxygen species are involved in a wide variety of diseases including ischemia-reperfusion injury, cancer, and various types of inflammation. Acute injuries such as cold ischemia in donor biopsies were associated with oxidative stress. Kidney allograft biopsies showed that oxidative stress levels were generally elevated in all biopsies regardless of diagnosis, but not significantly. The levels also were elevated in pretransplant biopsies. This study showed that oxidative stress is involved during acute allograft dysfunction. However, because of a lack of control biopsies, the results could not be interpreted effectively. Future studies involving kidney allograft biopsies must include a control sample from a normal kidney.

References


