Glutamine Donor Pretreatment in Rat Kidney Transplants with Severe Preservation Reperfusion Injury

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Background. Glutamine (GLN) has been shown to confer cytoprotection by enhancing endogenous heat shock protein (HSP) expression. We hypothesized that GLN donor pretreatment protects rat renal grafts against severe preservation reperfusion injury (PRI).

Materials and methods. GLN (0.75 g/kg) or saline was administered i.p. to male donor rats 24 h and 6 h before donor nephrectomy. Kidneys (n = 6/group) were cold-stored in UW solution for 40 h and transplanted into bilaterally nephrectomized syngeneic recipients. Grafts were removed after 24 h. Renal HSP 70 expression was determined by Western blotting. Graft function was assessed by serum creatinine. Renal cross sections were microscopically examined for acute tubular necrosis, apoptosis, tubular proliferation, and macrophage infiltration.

Results. GLN donor pretreatment significantly increased intragraft HSP 70 expression. Serum creatinine was not different between groups: 2.6 ± 0.2 mg/dL (saline) versus 2.7 ± 0.5 mg/dL (GLN). Both treatment groups showed severe tubular damage with significantly less papillary necrosis in the GLN group (P < 0.05). GLN significantly reduced the number of apoptotic tubular cells in the cortex, medulla, and papilla (P < 0.001 versus saline). Postinjury tubular proliferation, measured by PCNA antigen expression, and intragraft macrophage infiltration was not influenced by GLN.

Conclusions. In rat renal grafts suffering severe PRI pharmacological preconditioning with GLN attenuates early structural damage, especially tubular cell apoptosis. Stimulation of renal HSP 70 expression could be an important mechanism of GLN-induced cytoprotection. Our findings may have implications for the treatment of delayed graft function in recipients of marginal donor kidneys. © 2007 Elsevier Inc. All rights reserved.

Key Words: kidney transplantation; rat; ischemic injury; glutamine; heat shock protein; apoptosis.

INTRODUCTION

Kidneys from marginal donors have an increased incidence of delayed graft function (DGF). Clinically applicable donor pretreatment protocols to treat DGF in recipients of marginal renal grafts are not well established. We used a syngeneic rat kidney transplantation model with severe preservation reperfusion injury (PRI), induced by 40 h of cold storage, to test potential benefits of glutamine donor pretreatment.

Glutamine (GLN), an amino acid conditionally essential during critical illness and injury, has been shown to reduce cell and organ damage induced by endotoxemia or ischemia [1, 2]. As previously shown, glutamine induces endogenous heat shock protein 70 (HSP 70) expression in animals and humans, thus conferring cytoprotection against various stressors [3, 4]. Glutamine has been reported to improve outcomes in experimental models of intestinal and cardiac ischemia reperfusion injury (IRI) [2, 5]. In animal models of renal IRI, elevated HSP 70 levels have been associated with improved functional outcomes [6, 7].

In our present study, we hypothesized that glutamine donor pretreatment, initiated 24 h before organ
procurement, induced renal HSP 70 expression and attenuates early structural damage and functional impairment in 40 h cold-preserved syngeneic rat kidney grafts.

MATERIALS AND METHODS

GLN Administration and Experimental Design

GLN was administered as an alanyl-glutamine dipeptide (Fresenius-Kabi, Homburg, Germany), which was dissolved in saline. GLN solutions were filtered with a 0.45-μm filter before intraperitoneal administration. Donor rats received either 0.75 g/kg GLN or saline (n = 6 per group) 24 h and 6 h before left nephrectomy. Grafts were cold-stored in UW solution for 40 h and transplanted into bilaterally nephrectomized syngeneic recipients. In the sham operation group, animals (n = 3) underwent right nephrectomy and the contralateral kidney was used for additional experiments. Animals were sacrificed 24 h after surgery for blood sampling and organ harvesting. Kidneys were cross-sectioned and stored in preparation for Western blot analysis, conventional histology, and immunohistochemistry.

Animal Surgery

All animal protocols were reviewed and approved by the University of California San Francisco Committee on Animal Research, and animal care was in agreement with the National Institutes of Health (NIH) guidelines for ethical animal research (NIH publication number 80-123, revised in 1985). Kidney donors and recipients were inbred male Lewis rats (200 to 250 g; Charles River Laboratories, Wilmington, MA) housed under standard conditions with free access to food and water. All procedures were performed under inhalation anesthesia with isoflurane. Left kidneys were procured, flushed with ViaSpan, University of Wisconsin (UW) solution and stored at 4°C for 40 h.

Recipient underwent bilateral native nephrectomies followed by heterotopic kidney transplantation using an established microsurgical technique [8]. Briefly, end-to-side anastomoses between the renal vessels and the recipient’s abdominal aorta and inferior vena cava were created using continuous 8-0 nylon sutures; mean warm ischemia time was 17 ± 1.2 min. An end-to-end ureteroureterostomy was performed using interrupted 11-0 nylon suture. After recovery from anesthesia, animals were transferred to the housing facility and monitored until sacrifice 24 h post-transplantation.

Heat Shock Protein Detection

Renal cross sections including cortex, medulla, and papilla were snap frozen in liquid nitrogen and stored at −80°C until analysis. Western blotting was performed as previously described [1]. Blots were blocked with a 5% milk PBS Blotto solution. For HSP 70 detection, blots were incubated with a primary mouse-anti-HSP 70 antibody (Stressgen, Victoria, Canada). Blots were washed and incubated with a horseradish peroxidase-conjugated secondary goat-anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Densitometry was determined using the UVP Chemiluminescent Darkroom System (UVP Inc., Upland, CA).

Graft Function

Graft function was assessed by serum creatinine measurement 24 h post-transplantation. Recipient blood samples (0.5 mL) were processed by IDDEX Veterinary Services (Sacramento, CA).

Tubular Necrosis Score

For morphological studies, formalin-fixed renal grafts (n = 6 per group) were used. Paraffin cross sections (4 μm) were stained with hematoxylin and eosin and periodic acid-Schiff (PAS) using standard procedures. A renal pathologist, blinded for the experimental conditions, examined and scored kidney sections for acute tubular necrosis (ATN) as previously described [9]. ATN was graded as follows: 0 represented no abnormalities, and 1, 2, 3, and 4 represented slight (<20%), moderate (20 to 40%), severe (40 to 60%), and near-total (>60%) necrosis of the renal parenchyma, respectively.

Apoptosis Detection

Renal apoptosis was examined using the in situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN) based on the TdT-mediated dUTP nick end labeling (TUNEL) method. Paraffin-embedded renal cross sections including the cortex, medulla, and papilla were treated according to the manufacturer’s instructions and stained with Fluorescein. TUNEL-positive apoptotic cells were visualized under a fluorescence microscope (Zeiss Axio Imager A1, Jena, Germany) and quantitated using a digital imaging system (Zeiss AxioCam HR with Axiovision 4.4 software). In each microcompartment, 10 to 15 randomly chosen fields of view (FOV) per section were evaluated. The means of six samples were grouped together to obtain a mean ± SD. Positive cell staining was recorded digitally and expressed as the percentage of TUNEL-positive area per FOV at 400× magnification.

Immunohistochemistry

For assessment of intragraft monocyte/macrophage infiltration a monoclonal mouse anti-ED1 antibody (endothelial 1 antigen on monocytes/macrophages; Serotec, Oxford, United Kingdom) was used. For assessment of tubular cell proliferation a monoclonal mouse anti-PCNA (proliferating cell nuclear antigen, clone PC10; Zymed Laboratories Inc., San Francisco, CA) was used. Immunohistochemistry was carried out on 2 μm paraffin sections. ED1 staining was performed using the alkaline-phosphatase-anti-alkaline-phosphatase (APAAP) method on paraffin sections after deparaffinization and rehydration. Sections were incubated for 60 min at room temperature with primary Ab at 1:1000 dilution in RPMI (Seromed, Heidelberg, Germany) with 10% fetal calf serum (FCS) and 3% bovine serum albumin (BSA). Fast Red Kit (DakoCytomation, Hamburg, Germany) was used for detection and visualization.

PCNA staining was performed using the standard avidin-biotin-complex method (Dako). Sections were deparaffinized in xylol, rehydrated through graded alcohols and cooked in citrate buffer (pH 6.0) for 5 min. Slides were then incubated with primary Ab (diluted at 1:100) for 60 min at room temperature in Ab-diluent (Dako). AEC-chromogen (Dako) was used for visualization. Sections incubated with corresponding isotype controls instead of primary Ab were used as negative controls.

Intragraft ED1 and PCNA expression were evaluated in a blinded fashion. In the cortex, medulla, and papilla, 10 to 15 randomly chosen FOVs per section were evaluated using a light microscope (Zeiss Axio Imager A1, Jena, Germany). Positive cell staining was expressed as mean ± SD of cells per field of view (FOV).

Statistical Analysis

Values are expressed as means ± SD. If not otherwise indicated, groups were compared with the unpaired Student’s t-test. For com-
parison of graded variables (i.e., tubular necrosis score) the nonparametric Mann-Whitney U test was used. All tests were two-tailed. A P value of < 0.05 was considered to be significant. All statistical analyses were performed with the statistical package SPSS for Windows (Version 10.07; SPSS Inc., Chicago).

RESULTS

Renal HSP 70 Expression

Compared with saline, GLN pretreatment significantly increased HSP 70 expression in renal grafts suffering severe preservation reperfusion injury (P < 0.05; Fig. 1). In sham-operated controls (n = 3) renal HSP 70 expression was low. In this group, GLN treatment had no impact on renal HSP 70 expression 24 h after surgery.

Graft Function

In the sham group (right nephrectomy) mean serum creatinine on postoperative day 1 was 0.5 ± 0.1 mg/dL. Graft function was not different between saline and GLN pretreatment groups. The following serum-creatinine levels were measured 1 d post transplantation in recipients of 40 h cold-stored grafts: 2.6 ± 0.2 mg/dL (saline) versus 2.7 ± 0.5 mg/dL (GLN; P = not significant).

Tubular Necrosis Score

In sham-operated animals, no tubular necrosis was seen. Grafts transplanted after 40 h of cold storage showed severe tubular damage in the cortex and medulla, whereas the papilla showed moderate to severe damage (Fig. 2). Compared with saline, GLN donor pretreatment significantly reduced papillary necrosis (P < 0.05; Figs. 2 and 4). In the cortex and medulla of GLN pretreated kidneys, a trend toward less tubular necrosis was seen. However, differences between treatment groups were not statistically significant (Fig. 2).

Tubular Cell Apoptosis

Apoptotic tubular epithelial cells were detected using the TUNEL-method and visualized under a fluorescence microscope (Fig. 5). Sham-operated animals showed only a small number of TUNEL-positive apoptotic cells. Forty h of cold storage and transplantation induced marked tubular cell apoptosis in the cortex, medulla, and papilla (Figs. 3 and 5). Compared with saline, GLN pretreatment significantly reduced the number of apoptotic cells in the cortex, medulla, and papilla (P < 0.001; Fig. 3).
Tubular Cell Proliferation

Tubular cell proliferation was quantified by counting PCNA-positive cells. In sham-operated animals, only a few scattered PCNA-positive cells were detected. Forty h of cold storage and transplantation induced marked proliferative activity in the cortex and medulla, while no PCNA-positive cells could be detected in the papilla of either group (Table 1). GLN donor pretreatment had no influence on proliferation of tubular epithelial cells recovering from severe PRI (Table 1).

Macrophage Infiltration

One day after severe PRI, intragraft macrophage infiltration was evaluated by counting the number of ED1-positive cells per field of view (Table 2). No ED-1 positive cells were seen in native kidneys after sham operation. In the cortex and medulla of 40 h cold-stored grafts, ED1-positive cell counts were higher than in the papilla. Overall, GLN pretreatment had no effect on intragraft macrophage accumulation (Table 2).

DISCUSSION

To our knowledge, this is the first report on GLN-induced renoprotection in rat kidney transplants with severe ischemic injury caused by prolonged cold preservation. We found a strong antiapoptotic effect associated with GLN donor pretreatment. This effect was equally
seen in the cortex, medulla, and papilla of kidney grafts subjected to severe PRI. Ischemically damaged kidney grafts pretreated with GLN showed significantly elevated HSP 70 levels almost 3 d after the last GLN dose. Most studies investigating renoprotective effects of HSPs use warm ischemia models with clamping of the renal pedicle [10, 11]. In solid organ transplantation, however, the amount of structural damage and func-

### TABLE 1

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<th>Average Number of Intragraft PCNA+ Cells 1 Day Post-Transplantation*</th>
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<tr>
<td>Cortex</td>
<td>Saline (N = 6) 15.7 ± 3.8 GLN (N = 6) 15.9 ± 1.9</td>
</tr>
<tr>
<td>Medulla</td>
<td>Saline (N = 6) 13.3 ± 3.9 GLN (N = 6) 15.8 ± 2.3</td>
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<tr>
<td>Papilla</td>
<td>Saline (N = 6) 0 GLN (N = 6) 0</td>
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$P = \text{n.s.}$ between saline and glutamine treatment groups.

* Expressed as the number of PCNA (proliferating cell nuclear antigen)-positive cells per field of view at 400x magnification.

### TABLE 2

<table>
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<th>Average Number of Intragraft ED1+ Infiltrates 1 Day Post-Transplantation*</th>
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<tbody>
<tr>
<td>Cortex</td>
<td>Saline (N = 6) 3.7 ± 0.8 GLN (N = 6) 3.8 ± 0.5</td>
</tr>
<tr>
<td>Medulla</td>
<td>Saline (N = 6) 3.6 ± 0.7 GLN (N = 6) 3.4 ± 0.6</td>
</tr>
<tr>
<td>Papilla</td>
<td>Saline (N = 6) 1.4 ± 0.3 GLN (N = 6) 1.8 ± 0.9</td>
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$P = \text{n.s.}$ between saline and glutamine treatment groups.

* Expressed as the number of ED1-positive cells (monocytes/macrophages) per field of view at 400x magnification.

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FIG. 5. Fluorescein-stained apoptotic cells in representative cross-sections of 40 h cold-stored rat kidneys 1 d post-transplantation. Areas of confluent fluorescence in saline pretreated grafts (A), (C), (E) indicate the presence of large numbers of TUNEL-positive apoptotic cells, while GLN pretreated grafts (B), (D), (F) show little fluorescence. Quantification of TUNEL-positive cells, using a digital imaging system revealed a strong antiapoptotic effect of GLN donor pretreatment in all three microcompartments (see Fig. 3). Magnification × 200.
tional outcomes strongly correlates with the duration of cold ischemia. We used a life supporting syngeneic rat renal transplant model with bilateral native nephrectomy of the recipient. The choice of 40 h of cold preservation was based on an earlier study involving rat kidney transplants, where we showed that identical experimental conditions generated 1 wk recipient survival rates of 50% [12]. In rat renal transplants with prolonged cold storage, strong inflammatory responses and irreversible structural damage have been shown to occur early after reperfusion [9]. We therefore tested if elevated HSP 70 levels provide renoprotection during the first 24 h post-transplantation, probably the most critical phase of renal PRI.

Heat shock proteins (HSPs) are a highly conserved family of essential proteins and confer cellular protection against various forms of cellular injury, including ischemia-reperfusion, lung injury, and shock [3]. Short-term hyperthermia is known as one of the most potent inducers of endogenous HSPs. In a rat renal transplant model, enhanced HSP 72 expression, induced by donor hyperthermia, has been shown to protect against PRI [6]. However, in the clinical setting, short courses of hyperthermia as a means of HSP induction are impractical. In recent years, several compounds, some with established clinical applications, have been identified as inducers of renal HSP 70 expression [10, 11, 13]. For efficient pharmacological HSP 70 induction, however, some of these drugs require relatively high dosing. Therefore, their clinical safety remains a major concern. GLN, a conditionally essential amino acid, has been shown to efficiently induce HSP 70 expression in vitro and in vivo [2, 5]. In both animals and humans, GLN decreases end-organ injury and enhances survival in settings of sublethal stress [3]. Previous clinical trials indicate that even at high doses, GLN has no measurable side effects in healthy volunteers and critically ill patients [14, 15]. Our choice of GLN dosage and route of administration was based upon previous dose-response studies by Wischmeyer et al. [1], showing that a single infusion of 0.75 g/kg GLN rapidly increased GLN plasma levels and induced HSP 72 expression in heart and lung tissue in rats. The same authors also showed that a single intraperitoneal dose of 0.52 g/kg GLN, given 18 h before cardiac ischemia-reperfusion injury, decreased posts ischemic myocardial dysfunction [5].

In our present kidney transplant model, 40 h of cold storage induced severe tubular necrosis (ATN) and apoptosis, 24 h post-transplantation. GLN donor pretreatment significantly reduced papillary necrosis (Figs. 2 and 4), and slightly improved the ATN score in the cortex and medulla (Fig. 2). Quantification of PCNA-positive cells revealed marked proliferative activity of proximal tubules in the cortex and medulla (Table 1), despite severe PRI. GLN donor pretreatment did not dampen proximal tubular cell regeneration after severe ischemic injury.

Apoptosis of tubular epithelial cells is a common feature of ischemic and nonischemic renal injury and typically occurs at an earlier time point than tubular cell necrosis [16]. Over the past decade, therapeutic strategies to interrupt the cell death cascade have been the subject of intensive investigation. We herein demonstrate a strong antiapoptotic effect of GLN, when given to donor kidneys before cold ischemic injury and transplantation (Figs. 3 and 5). In vitro, limited extra-cellular GLN supplies are thought to modulate stress and apoptotic responses [17]. Whether beneficial effects of glutamine are purely HSP-dependent is still under investigation. Recent experimental data, however, suggest that on the molecular level, GLN-mediated cytoprotection strongly depends on the cellular capacity to activate an HSP response [18].

In a recent study using a rat model of hepatic warm IRI, pharmacological preconditioning with GLN failed to reduce intrahepatic neutrophil accumulation and did not improve functional outcomes [19]. Previously, Suzuki et al. showed that geranylgeranyl-acetone (GGA), an antiulcer drug, ameliorates acute renal failure via HSP 70 induction in a rat model of mild IRI [11]. Pharmacological preconditioning with GGA significantly improved renal function and reduced macrophage infiltration after warm IRI, generated by 30 min of renal pedicle clamping. Evidence was provided that beneficial effects of GGA were HSP 70-dependent. Using a rat renal transplant model with 40 h of cold ischemia, Redaelli et al. [6] showed that hyperthermia pretreatment markedly increased renal HSP 72 expression 8 h after transplantation. Hyperthermia pretreated grafts had a significant survival benefit and showed quicker functional recovery within the first week post-transplantation, compared with controls. These data suggest that induction of renal HSP expression may help to reduce posts ischemic renal dysfunction.

In the present study, donor pretreatment with GLN did not influence the number of intragraft monocyte/macrophage infiltrates (Table 2). Furthermore, renoprotective effects of GLN, as evidenced by reduced tubular necrosis and apoptosis, did not translate into improved graft function. Serum-creatinine levels of 2.7 mg/dL versus 1.1 mg/dL (Suzuki et al.) on postoperative day 1, clearly indicate substantial differences in the degree of renal injury, caused by either 40 h of cold storage and transplantation or 30 min of renal pedicle clamping [11]. The relatively short postoperative follow-up of 24 h is a limitation of our present study. Possible functional improvements, likely related to increased intragraft HSP 70 expression, could have become apparent with longer observation periods. However, critical events of PRI, decisively influenc-
ing long term graft function, typically occur within the first 24 h after transplantation [20, 21]. It is a known fact that extensive postischemic renal tubular injury results in early graft dysfunction. In a recent study using a comparable experimental setup, we showed that recipients of 39 h cold-stored rat kidney grafts with severe papillary necrosis, induced by mycophenolate mofetil treatment, had a 1 wk survival rate of 20%. In contrast, 1 wk survival in recipients of untreated grafts with moderate papillary necrosis was 90% [22]. Therefore, GLN-induced attenuation of post-ischemic structural damage, especially in the renal papilla (Figs. 2 and 3), may result in improved early graft function. In human kidney transplantation, the use of marginal donor kidneys is often associated with delayed graft function (DGF), resulting in poorer long-term outcomes [21]. Since our present renal transplant model uses “marginal” donor kidneys with severe PRI, it may be suited to develop strategies for the treatment of DGF.

Pharmacological HSP 70 induction in the donor kidney before severe ischemic injury, combined with HSP 70 stimulation in the recipient early after transplantation, may confer renoprotection and thus help to reduce the incidence of DGF. The fact that GLN induces durable HSP 70 expression, coupled with its proven benefits and safety in clinical trials involving critically ill patients, makes this compound ideal for the treatment of DGF.

CONCLUSIONS

Administration of GLN a few hours before donor nephrectomy protects rat renal grafts with severe PRI from subsequent structural damage, in particular tubular cell apoptosis. In the presence of prolonged cold storage, HSP 70 induction could be an important mechanism of GLN-mediated renoprotection. Our findings may have implications for the treatment of delayed graft function in recipients of marginal donor kidneys.

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REFERENCES