Ischemia Reperfusion Injury in a Neonatal Rat Renal Transplantation Model

Munetaka MORI, Tatsuya AOKI, Shin ENOSAWA*, Mitsufumi ENDO, Tomoyuki MIYASHITA, Akihiko TSUCHIDA and Yasuhisa KOYANAGI

Third Department of Surgery, Tokyo Medical University
*Department of Regeneration Surgery, National Research Institute for Child Health and Development

The bulk of renal transplants in Japan are cases of living related transplantation in adults. Nevertheless, for a variety of reasons, renal transplantation from pediatric donors has not been actively pursued and there has been virtually no fundamental investigation pertaining to pediatric renal transplants. In the present study, we investigated the characteristics of ischemia reperfusion injury to the immature kidney.

The animals used in experiments were female Sprague-Dawley rats weighing 250 g or 300 g, used as adult rats, and Sprague-Dawley rats 2 weeks old, used as neonate rats. First the right kidney was excised and then the left renal artery and vein were clamped for 1 or 2 hrs. Survival rates 24 and 48 hrs after reperfusion were measured, as were serum BUN, creatinine and TNF-α. Expression of TNF-α mRNA in kidney, small intestine, liver, lung and spleen were also measured. In the histological examination, DNA synthetic activity was investigated using hematoxylin-eosin staining and the bromodeoxyuridine (BrdU) labeling index.

24 hrs after 1-hr clamping, no significant difference in survival rate could be noted between the adult group and neonate group. On the other hand, 24 hrs after 2-hr clamping, a statistically significant difference was recognized between the two groups, with the survival rate at 78.6% for the adult group but only 16.7% for the neonate group. Whereas the survival rate 48 hrs after 2-hr clamping was 27.3% for the adult group, it was only 7% for the neonate group. The survival rate dropped sharply for both groups but no significant difference was recognized. Serum BUN and creatinine values increased in both groups but there was no significant difference. TNF-α in serum and tissue increased conspicuously only in the neonate group. In both groups, the BrdU labeling index increased more in rats undergoing ischemia reperfusion than in rats undergoing kidney excision alone.

The results indicate that in terms of survival rate and TNF-α, the neonate group as donor is somewhat inferior to the adult group. However, there are no differences between the two groups in serum BUN and creatinine. Because DNA synthetic activity is higher in the neonate group, it is believed that the group could serve amply as transplant donor if postoperative regeneration can be skillfully induced.

Key Words: Ischemia reperfusion injury, Renal transplantation, Neonatal rat, TNF-α

Introduction

In Japan, there are approximately 190,000 patients suffering from chronic renal failure. Roughly 14,000 of these patients are registered for renal transplantation, but the number of patients who undergo renal transplantation each year is currently about 600 to 7006). Most of these transplants, however, are from living relatives, while approximately 160 to 200
Ischemia Reperfusion Injury in a Neonatal Rat Renal Transplantation Model

other cases are transplants from cadaver or brain dead donors. At present, most donors are adults, and there are virtually no transplants from pediatric donors. Among the reasons are social considerations such as the difficulty of coordinating the provision of potential pediatric donors and medical considerations including the fact that adequate renal functions cannot be expected from a pediatric kidney of small size with underdeveloped tissue, plus the fact that there are problems in surgical procedure such as blood vessel anastomosis, which can easily result in postoperative thrombogenesis. Nevertheless, renal transplants from pediatric brain-dead donors are being performed, although the number of such cases is small. In Japan in the year 2000, there were 78 patients waiting for pediatric renal transplantation, but only 2 patients actually underwent the procedure. By contrast, in the United States in 2000, there were 625 patients awaiting pediatric renal transplantation and 616 of these patients (approx. 99%) underwent the procedure. It is reported that 335 of these patients received a kidney from a living donor while the remainder (about 54%) received it from a dead donor7).

If approval can be gained in the near future for brain-dead donors aged under 15 years, opportunities for transplants from pediatric donors are expected to increase. But there has been no report on ischemia reperfusion injury to pediatric donor kidney. In the present study, therefore, neonate and adult rats were used as models for undergoing ischemia reperfusion measures and for investigating survival rate, renal injury and regenerative activity from injury.

Materials and methods

Animals

The adult rats were male Sprague-Dawley rats weighing 200 g to 300 g. The neonate rats used were born from Sprague-Dawley pregnant rats 12 to 16 days previously, weighing 20 g to 30 g (CLEA Japan, Inc., Tokyo, Japan). They were kept under a daily-controlled 12-hr light, 12-hr dark lighting cycle at 23°C and were given standard rat chow (CE2) (Japan CLEA, Inc., Tokyo, Japan).

Surgical animal models

Under general anesthesia with ether, both neonate rats and adult rats underwent open laparotomy via median incision. The retroperitoneum was opened; the right renal artery and vein, plus ureter, were ligated, and the right kidney was resected. Next, the left kidney was exposed, and the left renal artery and vein were clamped for 1 or 2 hrs, then released or declamped. Experiments were conducted from 6 to 9 p.m. and evaluations were made 24 and 48 hrs later.

Experimental design

Three test groups were established as follows: 1) a group subject to right kidney resection only (KR group); 2) an ischemia reperfusion group in which the left renal artery and vein were clamped after right kidney resection (KR-IR group), and 3) a group in which there was no right kidney resection or ischemia reperfusion and no measures were taken (control group). Based on this, the following groups were established: among neonate rats, a KR-IR group (1-hr ischemia: n=26, 2-hr ischemia: n=31), a KR group (n=25) and a control group (n=5), and among adult rats, a KR-IR group (1-hr ischemia: n=8, 2-hr ischemia: n=13), a KR group (n=11) and a control group (n=5).

Postoperative evaluation

The survival rates of adult and neonate rats were investigated 24 and 48 hrs after measures...
were taken with each of the aforesaid groups. When the animals were sacrificed, blood samples were taken from the femoral artery and vein of neonate rats and the femoral vein of adults rats, and serum BUN, creatinine and TNF-α were measured.

In addition, RT-PCR and quantitative PCR were used to measure TNF-α mRNA in frozen specimens. Regarding RT-PCR, total RNA was extracted from the tissue by homogenization in ISOGEN (Nippon Gene, Toyama, Japan), and then isolated by precipitation with chloroform and isopropanol. The integrity of RNA was assessed by agarose gel electrophoresis and ethidium bromide staining. Five micrograms of total RNA was reverse transcribed at 42°C for 50min in the presence of 0.5μg oligo (dT), 0.2 nmol/l DTT, 5×first-strand buffer, 1 nmol/l dNTP mixture, and 200U of Moloney murine leukaemia virus reverse transcriptase (Gibco BRL, NY, USA). First-strand cDNA (30 ng) was amplified using 0.5U AmpliTag (Applied Biosystems, CA, USA) in a 10 μl reaction volume containing 5 pmol/l primer pair, 1.6 nmol/l dNTP, 10×PCR buffer (Applied Biosystems, CA, USA). The sequences of primer for rat TNF-α: sense primer, 5'-CCACACCGTCAGCCGATTT-3' and antisense primer, 5'-GGCCACTACTTCAGCATCTCGT-3'. The amplifying conditions were 35 cycles of the following: denaturation for 30 s at 94°C, annealing for 30 s 60°C, and extension for 30 s at 72°C. The PCR products were analysed in a 1% agarose gel stained with ethidium bromide. Glyceraldehyde phosphodehydrogenase (GAPDH) gene expression served as the loading control.

Rat TNF-α 4319411T (PE Biosystems, CA, USA) TagMan Universal PCR Master Mix 4304437 was used as quantitative PCR and measurements were taken with ABI Prism (r) 7700 Sequence Detection System. Similarly, in the neonate KR-IR group, measurements were taken of TNF-α mRNA expression in lung, liver, spleen and small intestine.

In histopathological examination, thrombogenesis and other tissue changes were observed after staining the resected left kidney with hematoxylin–eosin (HE). In addition, 1 hr prior to sacrifice, bromodeoxyuridine (BrdU) 100 mg/Kg was administered in the inraabdominal cavity, the left kidney was resected, prepared as frozen section and stained with anti BrdU antibody. The BrdU labeling index was calculated as the ratio of BrdU positive cells to all cells. The average of five microscopic fields (magnification×100) was taken. The control group, the KR group (24 hrs after resection), and the KR-IR group (24 hrs after 1-hr clamping) were compared both in the neonate group and in the adult group.

**Statistical analysis**

Survival rate was evaluated by chi-square test and the Mann–Whitney U-test was used for blood biochemistry tests. A p-value of less than 0.05 was considered to indicate a statistically significant difference.

**Results**

**Survival rate**

No significant differences were noted in survival rate in the KR-IR group (1 hr) after 24 and 48 hrs. Nor was a significant difference recognized in the KR-IR (2 hr) group after 48 hrs. In the KR-IR (2 hr) group, however, whereas the survival rate after 24 hrs in the adult rats was 11 of 14 cases (76.8%), it was only 5 of 18 cases (38.4%) in the neonate rats. Thus a statistically significant difference could be recognized between the two groups (p=0.0057) (Table 1).

**Serum BUN and creatinine**

In the neonate KR-IR group with 2-hr clamp-
Ischemia Reperfusion Injury in a Neonatal Rat Renal Transplantation Model

Serum and tissue TNF-α

In the neonate KR-IR group with 1-hr clamping, serum TNF-α was 3089.6±447.9 pg/mg, and in the adult KR-IR group with 1-hr clamping, it was 15.1±15.4 pg/mg. The neonate group thus exhibited a significantly high value (p=0.0126) (Fig. 2).

In all the neonate rat groups, there was TNF
expression 30 min after reperfusion. Roughly equivalent expression was found in the KR group and control group of adult rats. There was virtually no expression in the KR-IR group with 1-hr clamping 30 min after reperfusion. In a comparison of neonate rats and adult rats, it was found that whereas expression was strong in neonate rats with 1-hr clamping 30 min after reperfusion, expression was virtually absent in adults rats. In all other groups, expression was strong in neonate rats as compared with adults rats (Fig. 3).

In the neonate KR-IR group with 1-hr clamping, there was TNF-α mRNA expression in lung, liver, spleen and small intestine (Fig. 4).

Pathological findings

HE staining revealed that in normal kidney of neonate rats, as compared to adult rats, glomeruli and other renal tissues were underdeveloped. After ischemia reperfusion, however, there was no significant difference between the two groups in thrombogenesis.

BrdU staining in neonate rats showed that DNA synthetic activity increased in the KR-IR group as opposed to the KR group and that the longer the ischemia period, the greater the increase. The BrdU labeling index was 25.5%
in the neonate KR-IR group with 1-hr clamping and 12.5% in the adult counterpart. In the neonate KR group, the index was 9.7%, and in the adult KR group, it was 3.21%. It was 0.61% in the neonate control group and 0.01% in the adult control group. In each case, staining was stronger in the neonate rat (Fig. 5).

**Discussion**

Success in renal transplantation depends on a number of factors including donor kidney function, warm or cold ischemia period, perfusion status, and the extent to which post-transplant ischemia reperfusion injury and rejection can be suppressed. These factors have been widely investigated in adults, but thus far, virtually no fundamental studies have been performed on pediatric renal transplantation. In the present study, ischemia reperfusion injury in neonate rat kidney was investigated.

Over the 10-year period from 1983 to 1994, only 42 cases of pediatric renal transplantation from donors less than 15 years old were performed in Japan, and thereafter, the mean number of renal transplants per year has been slight at about 10 cases. According to a report by the Japan Organ Transplant Network, over the 5-year period from 1997 to 2001 transplant kidneys were obtained from 16 brain-dead or cadaver donors at or less than 15 years of age, and these were transplanted into 31 patients. Donor ages ranged from 1 to 15 years (mean at 8.4 years) and recipients ranged from 6 to 60 years of age (mean age at 21 years). It is reported that in 24 of the 31 transplant cases (77.4%), the organ took and the patient could be taken off dialysis, while in the remaining 7 cases (22.6%) the organ did not take. There were 3 cases in which the youngest donor aged was 1 year, and the recipients were 2 adults and 4 children. The organs took in all but one of the recipients, who was 11 years old. In addition to these, Haba et al. reported another case of pediatric cadaver kidney in which the kidneys from a female donor aged 2 years and 10 months were transplanted into adults 47 and 49 years of age. Ito et al. reported a case in which a kidney from a 14-year-old male donor

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Fig. 5  BrdU staining in neonate rats and adult rats (×100).  

A: adult control, B: adult KR (24 hrs after resection), C: adult KR-IR (24 hrs 1 hr clamping)
was transplanted into a 12-year-old male recipient. There have been few reports on renal transplantation from pediatric donors, but the aforesaid results suggest that although there is not enough evidence yet to withstand evaluation, neonate kidney donors could be acceptable clinically.

In our study, no significant differences in survival rate were found between neonate and adult rats except for a significant difference in the survival rate of the KR-IR group (2-hr) after 24 hrs. In terms of serum BUN and creatinine, there was no significant difference between neonate and adult rats in extent of renal injury. In the KR-IR group (2-hr) after 48 hrs, because the majority of adults rats died, as did the majority of neonate rats, there is no doubt that 2-hr ischemia involves a high level of stress also in adult rats, but it was demonstrated that the neonate rat is more susceptible to stress. In recent years, changes in inflammatory cytokines have been observed as indices of in vivo changes due to various forms of stress, including inflammation or surgery. TNF-α is a nonspecific inflammatory mediator that finds expression during ischemic reperfusion; it is expressed during the post-transplant acute stage and is believed to cause inflammatory injury. In the injury mechanism, there is direct action by free oxygen radicals, for instance, through the activity of phospholipase A2 and action against vascular endothelial cells. In the latter action, the following three activities can be considered: 1) prostaglandin or platelet-activating factor is produced, causing injury or morphological change to vascular endothelial cells; 2) blood coagulation is promoted locally through increase of procoagulant activity, suppression of protein C anticoagulant, and suppression of plasminogen activator, and 3) expression of adhesive factors locally collecting cells that contribute to inflammation and activating these cells. In the present study, serum TNF-α rose significantly in neonate as compared with adult rats. What is more, in all neonate groups TNF-α mRNA was expressed in liver, lung, small intestine and spleen. This suggests that in neonates, ischemia reperfusion injury affects not only the kidney but all organs throughout the body, and that it has an impact on mortality rate. These results indicate that even if there is no change in the capacity of donor kidney per se, the systemic impact of post-transplant ischemia reperfusion injury is higher in neonates than in adults. In pediatric renal transplant, therefore, it is not only donor systemic conditions and improvement of donor organ preservation methods and of surgical techniques that are important; reducing ischemia reperfusion injury is also a crucial concern. For this reason, various types of drugs have been administered from early on in an attempt to reduce ischemia reperfusion injury. In recent years, research targeted at TNF-α has been inaugurated. Oyano et al. conducted experimental heart transplants on dogs in order to investigate the effects of anti TNF-α antibody. As a result, it was reported that the group given anti TNF-α antibody was favorable in comparison with the control group in terms of post-transplant cardiac function and histopathology. In addition, Kitada et al. created a renal ischemia reperfusion model using dogs and administered anti TNF-α antibody. It was reported that in all aspects, including renal function, renal blood flow volume, and histopathological findings, injury was reduced in the group given anti TNF-α antibody as compared with the control group. The aforesaid research points to the possibility that suppression in the production of TNF-α, an inflammatory cytokine, can improve results in pediatric renal transplantation.

On the other hand, it has also been reported
that TNF-α promotes hepatocyte propagation in the liver. In this action mechanism, TNF-α is produced from macrophages after liver excision or inflammation, and IL-6 expression is accelerated via TNF-α receptor 1 (TNFR1)/NF-κB of monocyte/B cells. This IL-6 accelerates expression of the initial gene group, for example, via IL-6 receptor/STAT3 of hepatocyte, and thus supports hepatocyte propagation. In order to investigate the action of TNF-α, Kimura et al. performed a 70% partial hepatectomy using rats and TNF-α production inhibitor was administered. It was reported that in the group given TNF-α production inhibitor there was a significant drop in the ratio of remnant liver per body weight and in DNA synthetic activity using BrdU. On the other hand, Tsutsumi et al. created a 90% liver excised rat model and reported that liver protective action and liver regeneration were promoted by using anti TNF-α antibody. These two experiments exhibit a mutually repulsive effect, and they suggest the possibility that there is a biphasic effect in TNF-α action, depending on the extent of stress. In our investigation, DNA regeneration by BrdU labeling index exhibited higher levels of staining in each group in neonate as opposed to adult rats. Higher TNF-α production was also recognized in neonate rats, and from these results, it was conjectured that TNF-α might upregulate kidney regeneration. Details are unclear, however, because there have been virtually no studies done on how TNF-α relates to cell propagation. In the present study, moreover, as compared with adult rats, in neonate rats no significant difference was found in thrombogenesis after ischemia reperfusion, even though neonate renal tissue is underdeveloped. These findings indicate that in neonate rats there are large fluctuations at the molecular level, as for example in cytokines, but that they do not manifest impacts at the macroscopic level. It will be necessary to achieve a good balance between reduction of renal ischemia reperfusion injury and promotion of regeneration by elucidating the biphasic action of TNF-α.

In conclusion, it was found that in neonate rats as compared with adult rats, serum TNF-α exhibits significantly high values after renal ischemia reperfusion, and it is suspected that this might impact on cell injury. Nevertheless, there were no major differences histologically or in renal function in terms of blood biochemistry. This indicated that neonates can function as donors even though the kidney is young and weak. What is more, the potential for propagation of renal tubule cells was more active in neonate rats, and it was concluded that if postoperative regeneration can be skillfully induced, neonates can be used amply as donor source. Basic research such as this is expected to expand the possibilities for pediatric renal transplantation and to yield improvements in the survival rates of renal transplantation cases involving neonate donor kidney, as well as improvements in therapeutic results.

References

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