**Immunologic role of nitric oxide in acute rejection of golden hamster to rat liver xenotransplantation**

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**AIM:** To evaluate the immunologic role and expression significances of nitric oxide (NO), nitric oxide synthase (NOS), and its isoenzyme in acute rejection to liver xenografts from golden hamster in rat.

**METHODS:** Liver transplantations were randomly divided into five groups (n=6-9): isografts (group I); xenografts (group II); xenografts plus cyclosporine treatment (group III); xenografts plus cyclophosphamide treatment combined with splenectomy (group IV); and xenografts using cyclophosphamide in combination with splenectomy (group V). The levels of ALT, TNF-α, and nitric oxide production (NOx) in serum of recipients were examined, and expressions of type I (iNOS) and type II (cNOS) nitric oxide synthase (NOS)-inducible NOS (iNOS) and constitutive NOS (cNOS) were observed by NADPH diaphorase histochemical and immunohistochemical staining.

**RESULTS:** The level of serum ALT, activity of serum TNF-α and systemic levels of NO metabolite in groups II and IV were higher than those of groups I and V (serum ALT, 2416 ± 475, 2540 ± 82.5 nkat.L⁻¹ vs 556.8 ± 43.5, 677.3 ± 38.2 nkat.L⁻¹; P < 0.01; serum TNF-α, 35.3 ± 16.1, 444.6 ± 28.1 ng.L⁻¹ vs 38.5 ± 5.2, 52.0 ± 5.7 ng.L⁻¹; P < 0.01; serum NOx 514.6 ± 18.1, 336.0 ± 43.0 nmol.L⁻¹ vs 26.1 ± 5.7, 27.7 ± 6.0 nmol.L⁻¹; P < 0.01). Cyclosporine in group III can repress the cellular immune response and the synthesis of nitric oxide and the expression of NO synthase, but not prolong the liver xenograft survival. The over-expression of NOS, iNOS and cNOS in liver xenograft rejection in groups II and IV were detected by NADPH diaphorase histochemical and immunohistochemical staining.

**CONCLUSION:** The degrees of acute rejection can be effectively repressed in golden hamster to rat liver xenografts with splenectomy and cyclosporine. Nitric oxide metabolites, nitric oxide synthase and its isoenzymes, above all inducible NOS (iNOS) can be used as potential diagnostic markers for acute rejection in liver transplantation. The cellular localization of nitric oxide varies according to the immunologic status of liver xenografts, thus thinking that hepatocyte-derived nitric oxide may be protective in the hyporesponsive state, but hepatic injury is likely triggered by centrilobular iNOS expression in the superresponsive state.

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**INTRODUCTION**

Immune rejection remains an impediment to its overall clinical success in orthotopic liver transplantation, and acute rejection is seriously harmful to the grafts and recipients. Besides clinical symptoms and signs, histological analysis of biopsy remains the most useful tool for assessing the severity of rejection, but the liver graft may have been severely damaged at this time. It is an urgent issue of the moment to study the mechanisms of acute rejection and search for special early markers of diagnosis in liver transplantation. The cellular immunity has been testified to be a chief mechanism in rejecting liver transplantation.[1-8]

In recent years, as an endothelium-derived relaxing factor (EDRF), nitric oxide (NO), which is a highly reactive free radical with a multitude of organ specific regulatory functions, has received a tremendous amount of attention within the realm of organ transplantation,[9-11] for it can induce directly specific and nonspecific immunity of the body.[12] While a great deal of research has centered upon its cellular and molecular biology and pharmacology, little is known about NO’s contribution to overall organ physiology or pathophysiology. In organ transplantation, NO has been postulated to possess immunomodulatory functions. However, it remains unknown whether NO is immunosuppressive or immunostimulatory. In addition, does NO have a role in immunologic function within the settings of acute rejection, chronic rejection, or hyporesponsiveness? Our previous studies suggested that the beneficial effects of hepatic nitric oxide on the reperfusion injury in the rat orthotopic liver transplantation (OLT) by supplementing NO pathway or inhibiting the endothelium NO with NG Nitro-L-arginine methyl ester (L-NAME).[13-15] It was reported that hepatocyte-derived NO may be protective in the hyporesponsive state. However, Jsoe et al.[16] reported recently that a selective iNOS inhibitor attenuated ischemia-reperfusion injury in the pig liver, suggesting that NO produced by iNOS has a cytotoxic rather than a hepatoprotective effect on the hepatic warm I/R injury. As a result, further investigation regarding the immunologic role of NO in acute rejection in OLT is warranted. Our aim was to investigate the immunologic role of nitric oxide and expressing significance of NOS and its isoenzyme in acute rejection of golden hamster to rat liver xenografts.

**MATERIALS AND METHODS**

**Materials**

Male Wistar rats weighing 180-260 g purchased from Shanghai Experimental Animal Centre, Chinese Academy of Sciences,
and female golden hamsters weighing 150-200 g, purchased from the Shanghai Municipal Institute of Family Planning were used as donors and recipients respectively. All animals were maintained on a 12 h light/dark cycle and fed commercially available rat chow and had free access to water. Nitroblue tetrazolium and β-NADPH reduced nicotinamide adenine dinucleotide phosphate, rabbit antionmouse iNOS(NOS[II]) and cNOS (NOS[III]) polyclonal antibody were obtained from Sigma.

Methods
Experimental Design All animals were randomly divided into five groups(n=6-9);Group I (isografts) both donors and recipients were Wistar rats; Group II, in which Wistar rats and golden hamsters served as donors and recipients respectively, is xenografts in acute rejection;Group III was subjected to orthotopic liver xenotransplantation treated with cyclosporine (30 mg/kg.d), served as cellular immunosuppressive group; Group IV was xenografts using cyclophosphamidine (40 mg/kg.d) in combination with splenectomy, as humoral immunity defeat group; and group V was xenografts using splenectomy,cyclosporine and cyclo-phosphamidc as double imminosuppressive group.

Orthotopic liver transplantation Orthotopic liver transplantation (OLT) was performed according to Harirha's three Cuff technique with minor modifications as previously reported[17,18], in which the suprapheptic vena cava (SVC) was reconstructed by the Cuff method, along with the infrahepatic inferior vena cava (IVC) and the portal vein. The bile duct was internally stented with a polyethylene stent. The splenectomy was simultaneously carried out in the grafted recipients.

Specimen measurement The blood samples were obtained via the tail vein at the days 3, 5, 7, 10, 14, and so on postoperatively, or via portal vein or infrahepatic IVC in the recipient being killed or its liver tissue being biopsyed, and then centrifuged by 3 000 r·min⁻¹ at 4 °C for 10 min. The upper serum after snap-frozen was immediately stored at -80 °C until cutting small amount of cellular infiltrates in sinusoid areas by the 3

Histopathology Sections of the grafted liver were fixed in 40 g·L⁻¹ paraformaldehyde and 4 g·L⁻¹ picric and in 0.1 mol·L⁻¹ sodium phosphate buffer, pH7.4, for 4 h at 4 °C. Subsequently, sections were frozen at -80 °C until cutting the sections. Cryostat sections were immersed for 10 min in 0.1 mol·L⁻¹ phosphate buffer,pH8.0, and were incubated for 40 min at 37 °C in prewarmed solution consisting of 0.1 mol·L⁻¹ phosphate buffer, pH8.0;0.3 g·L⁻¹ Tritox X-100;0.5 mmol·L⁻¹ nitroblue tetrazolium; and 1.0 mmol·L⁻¹ NADPH. After washing in 0.1 mol·L⁻¹ phosphate buffer,pH7.4 the sections were dehydrated with graded alcohol(70,80,95 and 100 mL·L⁻¹). Slides were rinsed in PBS and counter stained with fast red for 2 min, and cover slips were mounted on microscopic glass slides. Areas with a positive reaction for NADPH diaphorase were stained dark blue in cytoplasm, and in red nucleus[19-23].

Immunohistochemistry Immunohistochemical methods were used to detect the expressions of inducible NOS (iNOS) and constitutive NO synthase(cNOS) with specific polyclonal antibody against cNOS or iNOS by the avidin-biotin complex method using an ABC immunostaining kit (Vector Labs, Burlingame, Calif.).Areas with a positive reaction were stained pale brown.

Statistical analysis Data are presented as means ± standard errors of the means (±SEM).Comparisons among different groups of samples were made by two-tailed test χ² test and F test. A value of P<0.05 was considered to be statistically significant.

RESULTS
Survival
The Survival of recipient in groups II,III and V, in which no significant alteration was found(6.9±0.4, 7.3±1.0,7.0±0.6, d, respectively) significantly lowered as compared with that of groups I and V, the difference being not statistically significant(48.5±20.7d vs 37.1±9.9d, P>0.05,Table 1).

Biochemical parameters
Following OLT, serum samples were assayed for ALT,TNF-α and NO on 3, 7 and 14 postoperative days (Table 1).The serum values for ALT and TNF-α in groups II and IV were 4-11 times greater than those of groups I and V (P<0.01, vs groups I and V).The serum levels of NO metabolites (NOx) in groups II and IV were 12-20 times greater than in groups I, III and V.

Immunohistochemical stains for NO synthase (NADPH diaphorase staining)
The grafted liver specimens were fixed in 40 g·L⁻¹ paraformaldehyde and 4 g·L⁻¹ picric and in 0.1 mol·L⁻¹ sodium phosphate buffer,pH7.4,for 4 h at 4 °C. Subsequently,specimens were frozen at -80 °C until cutting the sections. Cryostat sections were immersed for 10 min in 0.1 mol·L⁻¹ phosphate buffer,pH8.0, and were incubated for 40 min at 37 °C in prewarmed solution consisting of 0.1 mol·L⁻¹ phosphate buffer, pH8.0;0.3 g·L⁻¹ Tritox X-100;0.5 mmol·L⁻¹ nitroblue tetrazolium; and 1.0 mmol·L⁻¹ NADPH. After washing in 0.1 mol·L⁻¹ phosphate buffer,pH7.4, the sections were dehydrated with graded alcohol(70,80,95 and 100 mL·L⁻¹). Slides were rinsed in PBS and counter stained with fast red for 2 min, and cover slips were mounted on microscopic glass slides. Areas with a positive reaction for NADPH diaphorase were stained dark blue in cytoplasm, and in red nucleus[19-23].

Histopathology
Histological examination of the grafted liver in groups I and V revealed almost normal liver sinusoidal architecture with the exception of complication of the secondary infections such as subhepatic abscess of cholangiojejunal fistula, pneumonia, etc. (Figures 1,2).Acute rejection appeared in heterogeneic groups II and V, respectively. On the 5th postoperative day, inflammatory cell infiltration was increased significantly by the 5th postoperative day; diffuse polymorphonuclear and mononuclear cell infiltration, massive necrosis of hepatocytes and hepatic parenchymal interstitial hemorrhage were found by the 7th postoperative day (Figure 3). In group II,cyclosporine decreased significantly cellular infiltration, but severe hepatocyte necrosis and cyclophosphamide greatly attenuated hepatic necrosis and interstitial hemorrhage,yet cellular infiltration still remained the principal feature. In group V of xenografts using splenectomy,cyclophosphamide and cyclosporine, the architecture of the hepatic lobule was well preserved, with no hepatocyte necrosis, and a small amount of cellular infiltrates only in the portal areas (Figure 4).
Figures 1, 2  Liver tissue from isograft (Group I) complicated with subhepatic abscess of cholangiojejunal fistula at 14 d post-transplantation and with pneumonia at 94 d post-transplantation. HE × 66

Figure 3  Liver tissue from acutely rejecting liver xenograft (Group II) at 7 d post-transplant. HE × 66

Figure 4  Liver tissue from xenograft (Group V) with double immunosuppressive action at 14 d post-transplantation. HE × 33

Figures 5, 6  NADPH diaphorase histochemical staining in acutely rejecting liver xenografts (Group II) at 2, 7 d post-transplantation. NADPH-d × 33, × 66

Figure 7  NO synthase in liver xenograft (Group V) with double immunosuppressive action at 14 d post-transplantation, showing negative NO synthase. NADPH-d × 33

Figures 8, 9  iNOS and cNOS in acutely rejecting rat orthotopic liver xenograft (Group II) at 7 d post-transplantation. ABC × 66

Figure 10  cNOS in liver xenograft (Group V) with double immunosuppressive action at 14 d post-transplantation. ABC × 33
NADPH diaphorase and immunohistochemical staining findings

Expressions of nitric oxide synthase (NOS) in grafted liver tissue were detected by NADPH diaphorase staining methods. A small amount of NOS positive expressions in group I was localized to vascular endothelium and hepatocytes. The expression levels of NOS in groups II, III, and IV were obviously increased by the 2nd postoperative day, dominantly localized to hepatocyte and hepatic sinusaloid lining cells. The expression time of NOS was earlier at least two days than that of pathological damage (Figure 5). The intensity of NO synthase staining by the 5th-7th postoperative day was strongest in both the portal inflammatory infiltrate and hepatocytes showing purple dark blue precipitation (Figure 6). In contrast, the intensity of NO synthase signal in group V was significantly weaker than that of groups II, III, and IV (Figure 7).

The immunohistochemical staining findings for grafted liver tissue demonstrated that a small amount of signal intensity of constitutive NO synthase was present in the grafted liver tissues in group I, but no expression of inducible NO synthase. In contrast, the intensity of both eNOS and iNOS, in groups II, III, and IV was significantly greater than in group I (Figures 8, 9). There was no significant difference in the staining intensity of both eNOS and iNOS between groups I and V (Figure 10).

DISCUSSION

Recently, the postoperative immunosurveillance after organ transplantation has received a tremendous amount of attention. It has been reported that the occurrence rate of rejection in clinical liver transplantation was still as high as 48-77%. The liver allotransplants do not undergo hyperacute rejection even if the liver is transplanted in a crossmatch positive or ABO mismatched recipient. Acquiring easily immunologic tolerance, livers grafted between widely disparate species can be more easily accepted than other grafted organs such as heart, kidney, etc., which were rapidly lost because of hyperacute rejection mediated by humoral immunity. The recipient of liver xenografts can even survive for days. Hamster-to-rat liver xenotransplantation (HORLT), as a concordant heterotopic liver transplantation, undergo acute rejection mediated by cellular and humoral immunity. Although the small amount of antibody titer was found in hamster-to-rat cardiac transplantation after recipient's splenectomy, the survival of recipient prolonged significantly. In contrast, the survival of liver xenograft failed to prolong significantly, though the antibody titer still was not high in hamster-to-rat liver xenotransplantation subjected to splenectomy. Our results demonstrated that the combined treatment with spenectomy and cyclophosphamide due to depressing humoral immune response could reduce significantly humoral antibody formation, and lighten the hepatocyte necrosis, and completely eliminate interstitial hemorrhage, but it failed to ameliorate infiltrating cells and the expression of NO synthase in liver xenografts, and prolong the xenograft survival. However, cyclosporine could obviously depress the cellular immunity to decreased cellular infiltration, but severe hepatocyte necrosis and hemorrhage remained unchanged, thus failed to prolong xenograft survival and to improve liver functions. Only the double immunosuppression of the combined treatment with spenectomy, cyclophosphamide, and cyclosporine completely repressed the rejection of liver xenografts, significantly reduced antibody formation and infiltrating cells, eliminated the grafted liver function and prolong xenograft survival. Nitric oxide (NO) is a highly reactive and commonly synthesized free radical with a multitude of organ specific regulatory functions. Within the realm of solid organ transplantation, NO has been the focus of attention. Discordant reports have appeared regarding the functional role of NO in systemic physiology and pathophysiology. In organ transplantation, elevated systemic levels of NO metabolites always accompany the acute rejection of heart, liver, renal, pancreas, and small bowel allografts in both humans and rats. The potential hepatoprotective or hepatotoxic effects of NO, however, have yet to be clarified, especially, the role of NO and sites of synthesis in the immunologic states following organ transplantation. Our preliminary studies confirmed that hepatocyte NO production may be hepatoprotective in state of free radical production in hamster-to-rat liver xenografts. Monitoring of NO levels has been suggested as a clinical diagnostic means for initiation of intervention in transplantation management. Nitric oxide synthesis is an important component of nonspecific defense synthesis for a number of pathogens. Until recently, the pathway for induction of iNOS was presumed to be initiated by macrophage cytokine elaboration or lipopoly-saccharide from gram-negative bacteria. The present knowledge suggests that specific and nonspecific immunity is mediated by iNOS. Therefore, nonreticulo-endothelial cells, such as hepatocytes, containing iNOS, may play an unrecognized role in immunity. The exact roles of NO in liver xenograft rejection are still not clear. Although NO, possessing diverse functions, such as regulation of local blood as an endothelium-derived relaxing factor, Inhibition of platelet aggregation, and attenuation of neutrophil adherence, as a natural extracellular scavenger of superoxide anions, NO was considered to have cytoprotective effects against the rejection of liver xenograft, and cytotoxic and cytostatic effector functions through the nitrosylation and inhibition of cellular enzymes critical to mitochondrial respiration and DNA synthesis. However, the role of NO in oxidative stress mediated injury, has been controversial. It was reported that iNOS mRNA in rat heart transplantation was present in the inflammatory infiltrate but not within the cardiac myocytes. In our study, the expression of iNOS in liver xenotransplantation was identified in both hepatocytes and portal inflammatory cells. Therefore, the exact role of NO in liver xenograft acute rejection remains to be further studied using both a selective iNOS inhibitor (aminoguanidine hemisulfate) and a relatively selective iNOS inhibitor. Recent studies demonstrated that intraportal administration of aminoguanidine hemisulfate, a selective iNOS inhibitor, significantly suppressed nitric oxide production and serum aspartate aminotransferase after reperfusion, inhibited nitrotyrosine expression and attenuated hepatic damage. Protective or injuring effects of NO may depend upon the relative local concentrations of NO and accompany of biologic modifiers such as IL-1, TNF-α, or INF-γ. The process of acute rejection, which may be organ specific with respect to its biochemical modulators, is determined not only by the properties of inflammatory infiltrates, but also by the response of the parenchymal cells within the specific graft. Our study also demonstrated that the serum levels of NO metabolites (NO2, NO3) in unmodified xenografts (group I) and xenografts using double-immunosuppressive action (group V). The cellular immunosuppressant using cyclosporine alone can repress the expression and synthesis of nitric oxide without improvement of graft survival. The efficacy of cyclosporine having the suppression of specific activated T cells, as an immunosuppressant for organ transplantation and severe refractory autoimmune diseases, increased its clinical
application. Our study revealed that cyclosporine treatment resulted in inhibition of iNOS expression and consequently reduces iNOS enzyme activity during acute liver xenograft rejection. Unfortunately, cyclosporine administration is associated with renal vasoconstriction and vascular injury, which is thought to be a major pathophysiological factor in chronic CSA-induced nephro-toxicity. Cyclosporine has been shown to generate superoxide through an as-yet-unclearified alteration of cytochrome P-450-dependent mixed function oxidases, the primary pathway of CSA metabolism. In *vitro*, superoxide has been demonstrated to enhance inactivation of NO released from endothelial cells. It was reported that NO maintains a protective function with vasoconstricting effect to CSA. In addition, NADPH diaphorase and immunohistochemical staining findings in this study indicate that nitric oxide synthase (NOS) and its isoenzyme, especially iNOS, can be used as potential diagnostic markers for acutely rejecting orthotopic liver transplantation. In conclusion, the degrees of acute rejection with double immunosuppressive action using splenectomy, cyclophosphamide and cyclosporine can be effectively repressed in golden hamster to rat liver xenografts. The elevated systemic levels of NO metabolites and the overexpression of NO synthase and its isoenzymes, especially iNOS, accompanying the acute rejection of liver xenotransplantation can be used as potential diagnostic markers for acute rejection. The cellular localization of nitric oxide varies according to the immunologic status of liver xenografts, thus hepatocyte-derived nitric oxide may be considered protective in the hyporesponsive state, but hepatic injury is likely triggered by centriflobular iNOS overexpression in the superresponsive state.

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