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Increased urinary nitrate excretion associated with hepatic allograft rejection in experimental rat models and clinical cases

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Abstract In this study, we investigated the relationship between excretion of urinary nitrate as a stable end-product of nitric oxide (NO) metabolism and hepatic allograft rejection. In experimental rat models, hepatic allograft rejection was associated with increased nitrate excretion with a peak on postoperative day 5. The severity of the hepatic allograft rejection was dependent on the increased urinary nitrate excretion. No significant increase in urinary nitrate excretion was observed in cases in which effective immunosuppression was achieved. Inducible nitric oxide synthase mRNA expression was upregulated parallel to interferon-γ gene expression in the graft-infiltrating mononuclear cells and spleen cells from the recipients. In clinical cases, urinary nitrate excretion increased parallel to increased serum cytosolic enzymes that accompanied rejection. These results suggest that urinary nitrate excretion is a useful indicator for the surveillance of graft rejection and the monitoring of therapeutic effects of antirejection treatments.

Key words Liver transplantation · Nitric oxide · Rejection

Introduction

Nitric oxide synthase, induced by cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α, produces large amounts of nitric oxide (NO) from L-arginine. NO is rapidly degraded to the stable end products: nitrite ($NO2^-$) and nitrate ($NO3^-$) [17]. Plasma nitrite levels and urinary nitrate excretion have been used as an index of NO production in vivo [14]. Langrehr et al. have demonstrated that plasma nitrate levels increased in host-versus-graft and graft-versus-host responses in rats [13]. Winlaw et al. have documented increased urinary nitrate excretion in cardiac allograft rejection in an experimental study [21]. In this study, we investigate the kinetics of urinary nitrate excretion in orthotopic liver transplantation under conditions of acute cellular rejection in an experimental rat model. We also examine the correlation between inducible NO synthase (iNOS) and proinflammatory cytokine expression. In addition, we evaluate the time course of urinary nitrate excretion and the rejection process in two representative clinical cases of steroid-resistant graft rejection.

Materials and methods

Experimental groups

Inbred male rats, weighing from 210 g–345 g, were used in all experiments. LEW and BN rats were purchased from Seac Yoshitomi (Fukuoka, Japan) and DA rats from SLC (Shizuoka, Japan). Liver grafting with rearterialization was performed according to the cuff method described by Kamada and Knoop [9, 10]. In order to examine various levels of graft rejection severity, we performed three different donor/recipient combinations as follows: (1) Group I (no rejection; $n = 5$): LEW donor (RT-1^l) into LEW recipient; (2) Group II (mild rejection group; $n = 5$): BN (RT-1^n) into LEW; (3) Group III (severe rejection group; $n = 5$): DA (RT-1^a) into LEW. As shown previously [5, 19], Group II was used as the spontaneously tolerant model because only a transient, mild rejection occurs and thereafter the graft survives permanently, while Group III was used as the acute rejection model. A fourth group was

treated with tacrolimus, provided as a crystallize powder (Fujisawa Pharmaceutical Co., Osaka, Japan) and dissolved in saline. Tacrolimus was administered *i.m.* at a dose of 1.3 mg/kg per day on postoperative days 3, 4, and 5 in the DA into LEW combination (Group IV; $n = 4$). All animals were weighed daily and maintained on a 12 h light/dark cycle in conventional animal facilities with water and commercial rat chow provided ad lib. Postoperatively, recipient rats were maintained in metabolic cages for daily urine collection. The urine volume from each recipient was measured daily, and urine samples for nitrate assay were stored at –20 °C after centrifugation. The experiments were reviewed by the Ethics on Animal Experiments committee of the Faculty of Medicine, Kyushu University and carried out under the control of the Guidelines for Animal Experiments of the Faculty of Medicine, Kyushu University according to the Law (No. 105) and Notification (No. 6) of the Japanese government.

Measurement of urinary nitrate excretion

Urine nitrate concentration in each sample was measured using an assay kit (Cayman Chemical, Ann Arbor, MI) according to manufacturer's instructions. Total nitrate excretion per 24 h was calculated and indicated as μmol/kg per day.

Isolation of liver infiltrating cells and splenocytes

The liver and spleen were surgically removed from the recipient animals and gently disrupted with a pair of glass slides. Liver infiltrating cells and splenocytes were isolated by centrifugation using Percoll solution (Sigma Chemical Co., St. Louis, MO). Cells at the 1.086 and 1.055 Percoll interface were pooled and washed in phosphate-buffered saline.

RNA extraction and RT-PCR of cytokine genes

Total cellular RNA was extracted from 1×10^7 graft-infiltrating mononuclear cells or from 1×10^7 spleen cells as described by Chomczynski and Sacchi [1]. RNA concentration was determined from the optical density at 260 nm. First-strand cDNA was synthesized from 10 μg total RNA using Superscript reverse transcriptase (Life Technologies, Gaithersburg, MD) with random primers according to the manufacturer's instruction. Aliquots of the resultant cDNA solutions were amplified by PCR with 2.5 U of Taq polymerase (AmpliTaq Gold, PE Applied Biosystems, Branchburg, NJ) and an appropriate pair of oligonucleotide primers in the presence of dNTP. At the first thermal cycle, the PCR samples were heated at 94 °C for 5 min, 54 °C for 2 min and then 72 °C for 3 min. The following thermal cycles were carried out as follows: 94 °C for 1 min, 54 °C for 1 min, and then 72 °C for 0.5 min. At the final cycle, the extension step was carried out at 72 °C for 4 min. The total number of thermal cycles was 30 for amplifying cDNAs of TNF-α, IFN-γ and iNOS and 25 for β-actin cDNA. PCR with β-actin primers of serially diluted samples was used to estimate the amount of cDNA. Appropriate dilutions of each sample were made to equalize the amount of cDNA. The nucleotide sequences of primers used in this study were as follows: TNF-α sense primer, 5'AGCACGGAAAGCATGATCCGAGATG-3'; TNF-α antisense primer, 5'GTTGTCTTTGAGATCCATGCCATTGG-3' [19]; INF-γ sense primer, 5' ATGAGTGCTACACGCCGCGTCTTTG-3'; INF-γ antisense primer, 5'GAGTTCATTGACAGCTTTGTGCTGG-3' [15]; iNOS sense primer, 5'AGAGTGAGAAGTCCAGCC-3'; iNOS antisense primer, 5'AGGCACACGCAATGATGG-3' [18]; β-actin sense primer, 5'ATGCCATCCTGCGTCTGGACCTGGC-3'; β-actin antisense primer, 5'AGCATTTGCGGTGCACGATGGAGGG-3' [15]. These primers were designed to specifically amplify cDNA fragments representing mature mRNA transcripts. The sizes of amplified PCR products were as follow: 374 bp (TNF-α), 405 bp (IFN-γ), 441 bp (iNOS), 607 bp (β-actin). Amplified PCR products were electrophoresed on a 1.8% agarose gel and visualized by ethidium bromide staining.

Patients

Two representative patients were a boy and girl, both seven years old, who were living-related liver transplant recipients with end-stage liver cirrhosis due to biliary atresia. Urine samples were collected daily until discharge. Both patients underwent an immunosuppressive regimen that included prednisone and trough-level-dependent doses of tacrolimus, so that therapeutic whole blood levels were maintained between 10 and 15 ng/ml. The method of measurement of urinary nitrate is described above.

Statistical analyses

All data are presented as a mean ± SEM, and the Mann-Whitney's U test was used to determine statistical significance. A P value < 0.05 was considered to be statistically significant.

Results

Experimental rat models

In Group I, the recipient rats were healthy and survived throughout the observation period. In Group II, the recipient rats developed a transient weight loss and mild lethargy by postoperative day 20. Thereafter they showed a gradual recovery from these signs and survived at least through the observation period. By contrast, in Group III, the average survival period after transplantation was 13.2 ± 1.6 days. However, Group IV exhibited prolonged survival times of more than 60 days. No evidence of rejection was detected on histological examination of the hepatic allografts from Group IV on postoperative day 5 (data not shown).

As shown in Fig. 1, the urinary nitrate excretion of the allografted animals (Group II and III) increased markedly on postoperative day 5 and then gradually decreased. The animals in Group III exhibited higher urinary nitrate excretion levels, compared with those in Group II (on postoperative day 5: Group III, 502 ± 88; Group II, 175 ± 47 μmol/kg per day; $P < 0.05$). A rapid increase in urinary nitrate excretion was observed in Group III animals on postoperative day 5, which was the peak level of excretion. The urinary nitrate excretion of Group I animals was much lower than that of Groups II and III throughout the observation period. The tacrolimus treatment in Group IV completely abol-

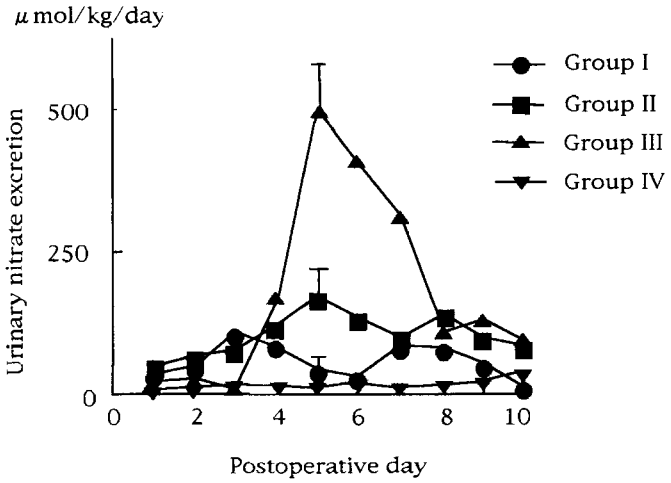

Fig. 1 Urinary nitrate excretion by Group I (syngeneic; $n = 5$), Group II (mild rejection; $n = 5$), Group III (severe rejection; $n = 5$) and Group IV (tacrolimus-treated; $n = 4$). Results are expressed as a mean ± SEM

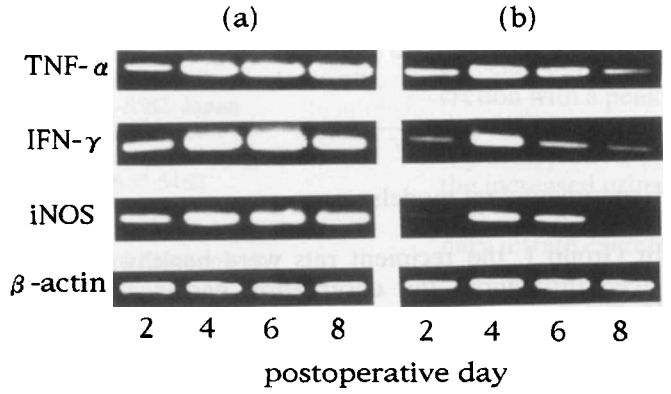

Fig. 2 Representative data of mRNA upregulation of proinflammatory cytokines, TNF-α and IFN-γ, and iNOS in (**a**) graft-infiltrating mononuclear cells and (**b**) spleen cells in Group III

ished the elevation of the urinary nitrate excretion observed in Group III ($P < 0.05$).

In the graft-infiltrating cells from Group III, the iNOS mRNA expression was upregulated parallel to both IFN-γ- and TNF-α mRNA expression on postoperative day 4 and 6, compared with those on postoperative day 2 (Fig. 2a). Thereafter, the iNOS and IFN-γ gene expression levels were downregulated on postoperative day 8, but the high TNF-α gene expression observed on postoperative days 4 and 6 was maintained to postoperative day 8. In contrast, the iNOS mRNA expression in the spleen cells from Group III was upregulated in parallel with both TNF-α- and INF-γ mRNA expression on postoperative day 4, and later, all three gene expression levels were gradually downregulated on postoperative days 6 and 8 (Fig. 2b). Thus, the upregulation of

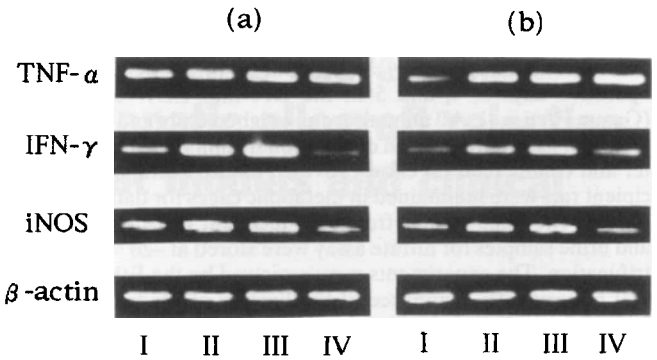

Fig. 3 Representative data of mRNA upregulation of proinflammatory cytokines, TNF-α and IFN-γ, and iNOS on postoperative day 4 in (**a**) graft-infiltrating mononuclear cells and (**b**) spleen cells in Group I, Group II, Group III, and Group IV

iNOS mRNA on days 4–6 after transplantation coincided with the elevation of the urinary nitrate excretion observed in Group III.

The expression of iNOS, TNF-α, and IFN-γ mRNAs in the graft-infiltrating cells and spleen cells from each experimental group on postoperative day 4 is shown in Fig. 3. Interestingly, iNOS- and IFN-γ gene expression in both graft-infiltrating cells and spleen cells was markedly suppressed by the tacrolimus treatment in Group IV rats, but the TNF-α gene expression was not significantly suppressed.

Clinical cases

In the clinical cases we examined the patients had no renal dysfunction as documented by the plasma creatinine levels throughout the observation period. In patient 1 (Fig. 4), the urinary nitrate excretion increased temporarily immediately after transplantation and gradually decreased by postoperative day 2. Thereafter, the urinary nitrate excretion began to increase again. The serum alanine aminotransferase (ALT) also began to increase on postoperative day 5. The elevation observed at these time points (on days 5–8) was resistant to steroid pulse therapy because the recipient was treated with methylprednisolone during that period. Percutaneous liver biopsy revealed acute cellular rejection on postoperative day 7. To suppress the cellular immune response associated with graft rejection, the patient underwent the OKT3 therapy from postoperative days 9–14 (2.5 mg/day), when urinary nitrate excretion showed a transient 8-fold increase on postoperative day 11. The OKT3 therapy was effective and immediately depressed the serum ALT. On postoperative day 28, the urinary nitrate excretion began to increase again, followed by an increase in the serum ALT. However, signs of graft re-

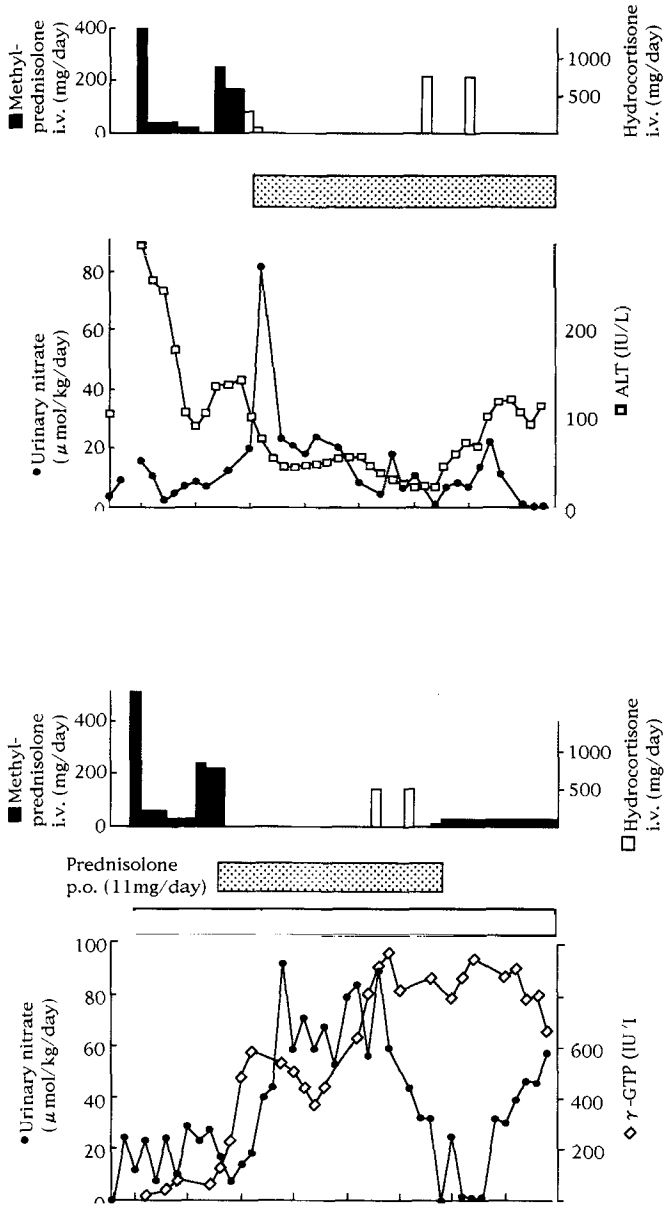

Fig. 5 Clinical course of urinary nitrate excretion in patient 2. Day 0 indicates the day of liver transplant

jection were readily depressed by the hydrocortisone therapy.

In case 2 (Fig. 5), the patient received tacrolimus and methylprednisolone in a routine manner after transplantation. However, tacrolimus was discontinued and cyclosporine A started on postoperative day 12 because of unstable trough levels of tacrolimus. The change of immunosuppression caused in a rise in the urinary nitrate excretion. Percutaneous liver biopsy on postoperative day 21 revealed a mild to moderate rejection, accompanied with a high serum γ-glutamyl transpeptidase (γ-GTP). The urinary nitrate excretion decreased temporarily with the administration of hydrocortisone. However, the urinary nitrate excretion began to increase again on postoperative day 34. Finally, the patient experienced repeated rejection episodes and the function of grafted liver was unstable.

Discussion

In cardiac allograft rejection, an increased NO production was detected by a rise in the excretion of nitrate as a stable metabolite of NO in an experimental study [21]. Plasma nitrite and nitrate have been reported to increase with allograft rejection and decrease with successful antirejection therapy in both experimental models and patients [2, 7, 13, 20].

In our experiments, the urinary nitrate excretion peaked on postoperative day 5 in both the mild rejection- (Group II) and the severe rejection (Group III) groups, as shown in Fig. 1. Group III showed a remarkably higher peak urinary nitrate on postoperative day 5 compared with Group II. Therefore, these results suggest that the increase in urinary nitrate excretion can be used as a suitable indicator of acute cellular rejection. Furthermore, higher urinary nitrate excretion levels were maintained in Group II throughout the observation period (data not shown), compared with Group I, but the urinary nitrate excretion began to decrease gradually with some transient and slight increases after postoperative day 5. The decrease in urinary nitrate excretion, which reached minimum levels around postoperative day 20, was correlated with improvement in rejection symptoms such as weight loss and lethargy (data not shown).

Various cytokines, such as IFN-γ, TNF-α, IL-1 and IL-2, are involved in the regulation of allograft immunity and activate iNOS [14]. Ding et al. demonstrated that IFN-γ alone could induce iNOS [3]. Our current study shows that the increase in TNF-α mRNA expression was not always accompanied by an increase in iNOS mRNA expression. In this study, both iNOS and IFN-γ gene expression levels increased on postoperative days 4 and 6 and decreased on postoperative day 8 in the graft-infiltrating mononuclear cells from Group III animals (the severe rejection group). By contrast, iNOS mRNA expression in the spleen cells from Group III animals peaked on postoperative day 4 and then decreased together with the mRNA expression levels of TNF-α and IFN-γ. These results suggest that after orthotopic liver transplantation iNOS mRNA expression is regulated mainly by IFN-γ but not TNF-α. Moreover, since iNOS mRNA expression in the spleen cells apparently increased on postoperative day 4, it suggests that NO is produced not only in the hepatic al-

lograft but also in peripheral lymphoid tissue after liver transplants.

Potential sources of NO production in-vivo include not only graft-infiltrating mononuclear cells but also endothelial cells and hepatocytes. A number of experiments have demonstrated the existence of iNOS in hepatocytes [4, 16]. Kuo et al. demonstrated that hepatocytes had high levels of iNOS mRNA expression in rejecting liver allografts [11, 12]. Although we have not examined the iNOS induction in the hepatocytes in the allografts, it is conceivable that hepatocytes produce NO and regulate the immune response of the recipients. It remains controversial whether NO generated during acute rejection is harmful or beneficial to the recipients. Hoffman et al. demonstrated that NO production during the response to alloantigens inhibits lymphocyte proliferation [6]. NO production may play important roles for the spontaneous acceptance of hepatic allografts. However, the function of NO in graft survival/rejection remains to be fully clarified in hepatic allografts.

In patients, urinary nitrate excretion correlates well with the rejection response to the hepatic allograft. However, urinary nitrate excretion did not decrease when the rejection was steroid-resistant.

In case 1, the OKT3 therapy increased urinary nitrate excretion by 8-fold. The administration of OKT3 is nearly always accompanied by a cytokine release syndrome characterized by symptoms such as fever, dyspnea, tremor, wheezing, headache, tachycardia, chills, and hypertension [8], In the case presented here, we presume that OKT3 administration induced the cytokine release syndrome and eventually resulted in the extraordinary and transient increase in urinary nitrate excretion. Thereafter urinary nitrate excretion levels dropped rapidly in response to successful depression of rejection response by the OKT3 therapy. It seems that the increase of urinary nitrate excretion preceded the rise of ALT as shown in Fig. 4. Increases in urinary nitrate excretion may be an earlier marker of rejection than rises in transaminase levels.

In case 2, changing immunosuppressive agents from tacrolimus to cyclosporine A resulted in a rise in urinary nitrate excretion and the occurrence of the rejection response. Devlin et al. [2] demonstrated that tacrolimus depressed plasma NO metabolites more effectively than cyclosporine A. The results observed in this case may support their observation.

In another patient, who experienced viral hepatitis without apparent rejection histology, urinary nitrate excretion increased parallel to the serum cytosolic enzymes. We could not distinguish hepatic allograft rejection from viral hepatitis by monitoring the urinary nitrate excretion as described in the present study.

In summary, the measurement of urinary nitrate excretion could provide a non-invasive marker for hepatic allograft rejection and an estimation of effects of anti-rejection therapy.

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