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European Journal of Pharmacology 391 (2000) 31–38

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Inhibition of inducible nitric oxide synthase improves graft function and reduces tubulointerstitial injury in renal allograft rejection

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Received 14 October 1999; received in revised form 27 December 1999; accepted 4 January 2000

Abstract

Increased levels of nitric oxide (NO) are found in rejecting renal allografts. Inducible NO synthase (iNOS) in infiltrating monocytes/macrophages could lead to NO bursts. NO may modulate the inflammatory response of early rejection due to its high reactivity with superoxide to yield peroxynitrite. To define the role of iNOS in acute renal allograft, rejection effects of the specific iNOS blockers iminoethyl-lysine and 7-butylhexahydro-1*H*-azepin-2-imine,monohydrochloride on renal function and morphology were investigated in renal allografts. Lewis rats received Brown Norway grafts with one kidney left in situ. All recipients were treated with low dose cyclosporine-A (2.5 mg/kg BW/day s.c.) to allow moderate rejection. In addition, one group received iminoethyl-lysine (10 mg/kg BW/day gavage) and one group received butylhexahydro-azepin-imine (3.4 mg/kg BW/day i.p.). Sham operated Brown Norway donor rats served as baseline controls. Compared to controls, low dose cyclosporine-A decreased glomerular filtration rate ($P < 0.05$) and numerically increased renal vascular resistance. Adding iminoethyl-lysine to cyclosporine-A improved renal hemodynamics. Adding butylhexahydro-azepin-imine to cyclosporine-A practically restored glomerular filtration rate and renal vascular resistance ($P < 0.05$) to control levels. Grafts treated with cyclosporine-A alone showed vascular, glomerular and tubulointerstitial lesions. Adding iminoethyl-lysine or butylhexahydro-azepin-imine to cyclosporine-A did not significantly reduce vascular and glomerular injury, but diminished tubulointerstitial injury as well as nitrotyrosine staining in tubular epithelium ($P < 0.05$). Thus, adding the iNOS blockers iminoethyl-lysine or butylhexahydro-azepin-imine to cyclosporine-A improved graft function and reduced tubulointerstitial lesions.

Keywords: Transplantation; Nitric oxide (NO) synthase; Inducible; Macrophage; Peroxynitrite

1. Introduction

Despite immunosuppression, acute rejection of the renal allograft still remains a problem for successful long-term graft survival. The free radical nitric oxide (NO) is considered an important agent in acute transplant rejection (Langrehr et al., 1992a,b; Stojanovic et al., 1996; Szabolics et al., 1998). Constitutive NO synthase (eNOS) in endothelium resulting in NO production at low concentrations antagonises platelet aggregation (De Graaf et al., 1992) and neutrophil adhesion (Gauthier et al., 1995). NO at high concentrations, however, is associated with pathophysiological responses due to avid binding to another radical, namely, superoxide (O_2^-) to form cytotoxic peroxynitrite ($ONOO^-$) (Beckman and Koppenol, 1996; Buttery et al., 1996).

Inducible NO synthase (iNOS) is responsible for production of large bursts of NO (Wever et al., 1997). iNOS can be expressed in several cells of the kidney: tubular epithelium, smooth muscle cells, and above all, in mononuclear inflammatory cells (Langrehr et al., 1993), that during acute rejection of a renal allograft infiltrate the kidney parenchyma. Circulating cytokines, such as interleukin-1 and tumor necrosis factor-α, of which release is increased in the rejecting kidney (Langrehr et al., 1992a,b; Mclay et al., 1994; Floege and Gröne, 1995; Takada et al., 1997), trigger iNOS expression. In fact, production of NO

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00021-2

was increased excessively during acute rejection, as assessed by plasma or urinary measurements (Winlaw et al., 1995; Worrall et al., 1995, 1996, 1997), and it has been postulated that NO synthesised by iNOS can enhance rejection processes.

Activated macrophages produce both NO and O_2^- radicals via activation of iNOS (Goto et al., 1997; MacMicking et al., 1997). Indeed, iNOS has recently been shown to be a peroxynitrite-generating enzyme (Xia and Zweier, 1997), leading to nitrotyrosine formation. Infiltrating monocytes and iNOS expression have been found to be co-localised in a kidney transplant model (Cattell et al., 1994; Heeringa et al., 1998). This suggests that several structural and functional features of acute rejection could be mediated by macrophages. However, the role of NO production by iNOS as a pathophysiological mediator of infiltrating mononuclear cells during acute graft rejection has not yet been specifically tested.

If iNOS is a mediator of the induction and progression of acute rejection, then selective iNOS inhibition may ameliorate histologic lesions as well as reduce transplant dysfunction. iNOS blockade may ultimately enhance prolonged long-term graft survival. In order to define the role of iNOS in acute renal allograft rejection, the effects of two different highly specific iNOS blockers, L-*N*6-(1-iminoethyl)-lysine-hydrochloride (Moore et al., 1994; Salvemini et al., 1995, 1996; Schwartz et al., 1997; Wolff et al., 1998) and 7-butylhexahydro-1*H*-azepin-2-imine-monohydrochloride [WO 95/11231, PCT/US94/11832] on renal function and pathology, were investigated in an allogenic rat kidney transplant model.

2. Materials and methods

2.1. Biological activity of iNOS inhibitors iminoethyl-lysine and butylhexahydro-azepin-imine

In vitro experiments confirmed that iminoethyl-lysine acts as a highly specific inhibitor of iNOS, reported to be approximately 30-fold more selective for iNOS ($IC_{50} = 3.3$ μM) than for eNOS ($IC_{50} = 92$ μM) or nNOS ($IC_{50} = 61$ μM) (Moore et al., 1994). Butylhexahydro-azepin-imine, prepared by chemical synthesis, has a 50-fold higher selectivity for iNOS ($IC_{50} = 0.55$ μM) than for eNOS and nNOS ($IC_{50} = 106$ μM) [WO 95/11231, PCT/US94/11832].

2.2. Rat renal transplant model

Left kidneys of male Brown Norway ($BN = RT1^n$) rats were transplanted heterotopically into male Lewis ($LEW = RT1^l$) recipients, with one native kidney in situ. The donors (180–210 g) and the recipients (300–350 g) were obtained from Harlan (Bicester, UK). Maintenance occurred under standard conditions (day/night 12/12; humidity 55%; temperature 22°C) with water and chow (RMH-TM rat chow; protein 22.2%; fat 4.8%; potassium 0.85%; sodium 0.40%, Hope Farms, Woerden, NL) ad libitum. The protocol was approved by the Utrecht University Committee for study in experimental animals.

2.3. Kidney transplantation

Donor nephrectomy, preservation, and heterotopic kidney transplantation procedures were performed as previously described by Fischer and Lee (1965) with some modifications (Stojanovic et al., 1996). The right donor kidney was perfused with and preserved in Custodiol (HTK-Tramedico, Alsbach) at 4°C. Cold ischemia was limited to 15 min. The vascular anastomoses were performed in an end-to-side-fashion using 8-0 nylon suture (Ethicon). Blood flow was restored to the graft within 30 min. After vascular anastomoses were checked for leakage, the ureter anastomosis was performed using 11-0 nylon suture in an end-to-end-fashion.

2.4. Experimental groups

The kidney recipients were divided into four groups (1–4).

Group 1 were sham operated Brown Norway rats for baseline values of the donor kidney prior to transplantation.

Groups 2, 3 and 4 had renal allografts and received a low dose of cyclosporin-A (2.5 mg/kg/day s.c.) to allow moderate vascular and interstitial rejection. Cyclosporine-A (Sandimune, Novartis Pharma, Basel, Switserland) was injected twice daily s.c. in half daily doses. The rats were weighed daily before administration of cyclosporine-A.

Group 3 was treated with cyclosporine-A and the selective iNOS-blocker iminoethyl-lysine. The dosage of iminoethyl-lysine of 10 mg/kg/day has been reported to effectively inhibit iNOS in vivo in the rat (Salvemini et al., 1995; Schwartz et al., 1997). Iminoethyl-lysine was administered twice daily by gavage in distilled water in half daily doses.

Group 4 was treated with cyclosporine-A and the iNOS-blocker butylhexahydro-azepin-imine in a dosage of 3.4 mg/kg/day [WO 95/11231, PCT/US94/11832]. Butylhexahydro-azepin-imine was injected i.p. twice daily, in half daily doses, diluted in saline.

2.5. Clearance measurements

Clearances of inulin (C_{in}) and para-aminohippuric acid (PAH) (C_{PAH}) were measured on day 7 post transplantation, using conventional techniques (Fransen and Koomans, 1995). Animals were anaesthetised with inactin. The left jugular vein was catheterised for infusions. The right femoral artery was catheterised for continuous measurement of mean arterial pressure and to draw arterial blood samples for determination of hematocrit and plasma con-

centrations of creatinine, inulin and PAH. We catheterised the ureter of the contralateral kidney while the transplanted kidney continued to drain into the bladder, in which a catheter was placed through a midline incision. After 1 h equilibration period, C_{creat}, C_{PAH}, C_{in} were measured during four 30 min urine collections to assess glomerular filtration rate and renal blood flow. At the start, midpoint and end of the clearance period, an arterial blood sample was drawn. Plasma and urine creatinine levels were determined colorimetrically (Sigma, St. Louis, MO). Inulin and PAH concentrations were measured photometrically with indoleacetic acid after hydrolysation to fructose and by a chromogenic aldehyde reaction, respectively.

2.6. Histology

Kidney slices were fixed in formalin or methacarn, embedded in paraffin, and stained with periodic acid Schiff (PAS) and trichrome. Sections were numbered and the pathologist was blinded to the code. Kidneys were evaluated for evidence of rejection and tubulointerstitial injury. The various types of injury were semiquantitatively scored from 0 to 3, with 0 indicating no pathological changes: 1, slight; 2, moderate; and 3, severe alterations. Vascular changes due to adhesion of inflammatory mononuclear cells to intima, thrombosis, or necrosis were evaluated for all preglomerular vessels per whole kidney section. Total vascular injury index was calculated as the sum of the severity scores (1, slight; 2, moderate; or 3, severe lesions) which were multiplied by the percentage of vessels displaying the concerning score. One hundred Glomeruli per kidney sections were evaluated for ischemic collapse, capillary obliteration by thrombosis, and mesangial matrix increase. Tubulitis was indicated by infiltrated mononuclear cells in the tubular epithelium, with score 1 indicating 4 cells per tubular cross-section, 2 indicating 4–9 cells, and 3 indicating more than 10 cells per cross-section. Tubular injury index was determined semiquantitively in 10 high power fields (40 × objective) with score 1 indicating mild lesions, 2 indicating moderate with cytoplasmic vacuolisation, and 3 indicating severe lesions with disruptions of basal membrane and loss of brush border. The interstitial injury index was semiquantitatively scored in 10 high power fields (40 × objective) with score 1 indicating occasional infiltration, 2 indicating focal infiltration, and 3 indicating entirely scattered infiltration of mononuclear inflammatory cells with more than 60% of the interstitial field covered.

2.7. Immunohistochemistry

Immunohistochemistry was carried out on 5 μm section of paraffin-embedded tissue. The monoclonal antibody ED1 (Serotec, Camon, Wiesbaden, Germany) was used on methacarn-fixed tissue at a dilution of 1:100 to demonstrate monocytes/macrophages. An alkaline phosphatase anti-alkaline phosphatase detection system was applied (Dako, Hamburg, Germany). The number of $ED1^+$-cells (monocytes/macrophages) was counted per glomerular cross-section in 100 glomeruli and also in 15 high power fields (40 × objective) of tubulointerstitium. A polyclonal immunoglobulinG rabbit antibody against nitrotyrosine (Upstate Biotechnology Bizol Eching, Germany) was used at a dilution of 1:20, to document peroxynitrite-induced modifications. Formaldehyde-fixed tissue was treated by microwave. A biotin-streptavidin-peroxidase detection kit supplied by Vectastain (Camon, Wiesbaden, Germany) was applied. Nitrotyrosine formation was evaluated semiquantitatively scored from 0 to 2, with 0 indicating no cytoplasmic staining, 1 pale staining and 2 profound intensity of staining in tubules of the inner cortex and outer medulla.

2.8. Statistics

One-way analysis of variance (ANOVA) on ranks (Kruskal Wallis), followed by the post-hoc Dunn's test was used to evaluate the data. $P < 0.05$ was considered significant. Data are presented as mean ± S.E.M.

3. Results

3.1. Follow up

Initial body weights were similar for all three groups of allograft recipients. In group 1 (consisting of sham operated donors), body weight was lower since animals were matched for donor kidney weight to compare functional and morphological parameters. In the first 3 days after transplantation, all animals lost weight. Cyclosporine-A treatment led to a slower decline of body weight than either cyclosporine-A + iminoethyl-lysine or cyclosporine-A + butylhexahydro-azepin-imine treatment, which were not significantly different ($P = 0.09$).

3.2. Clearance data of non-immunosuppressed groups

To assess the therapeutic value of selective iNOS inhibition with regard to graft function upon transplantation, we examined renal function of the renal allograft after 7 days of treatment with cclosporine-A alone, versus the iNOS blockers iinoethyl-lysine or bylhexahydro-azepin-imine in addition to cclosporine-A. Means of kidney weight and functional parameters are presented in Table 1.

To identify the hemodynamic effects of transplantation with cyclosporine-A alone, we evaluated graft function in comparison with normal function of donor kidneys prior to transplantation. Cyclosporine-A had no effect on mean arterial pressure and hematocrit values compared to controls. Transplantation and cyclosporine-A alone reduced glomerular filtration rate by approximately 50%. Renal

Table 1
Renal hemodynamic parameters before and after transplantation
Renal hemodynamics of transplanted kidney of immunosuppressed control recipients (CyA, cyclosporine-A) and iNOS blocked recipients (CyA + L-NIL, cyclosporine-A + iminoethyl-lysine; CyA + BAI, cyclosporine-A + butylhexahydro-azepin-imine) versus native donor kidney (BN, Brown Norway).
Data are mean ± S.E.M.; tested by one-way ANOVA on ranks (Dunn's method).
KW, kidney weight; MAP, mean arterial pressure; HT, hematocrit; C_{creat}, clearance of creatinine; C_{in}, clearance of inulin; RBF, renal blood flow; and RVR, renal vascular resistance.

	BN	CyA	CyA + L-NIL	CyA + BAI
n	6	7	7	6
KW g	0.82 ± 0.01	0.83 ± 0.03	0.84 ± 0.03	0.79 ± 0.03
MAP mm Hg	95 ± 4	90 ± 4	103 ± 4	91 ± 2
HT %	41 ± 2	42 ± 1	45 ± 1	42 ± 1
C_{creat} ml/min/g KW	0.91 ± 0.14	0.61 ± 0.10	0.50 ± 0.06	1.09 ± 0.24
C_{in} ml/min/g KW	1.19 ± 0.19	0.61 ± 0.13[a]	0.74 ± 0.09	0.96 ± 0.08
RBF ml/min/g KW	7.09 ± 1.09	4.57 ± 0.93	5.02 ± 0.76	7.50 ± 0.36
RVR units/g KW	15.5 ± 2.25	29.4 ± 7.00	25.3 ± 5.14	12.7 ± 0.47[b]

[a] $P < 0.05$ compared to BN.
[b] $P < 0.05$ compared to treatment with cyclosporine-A alone.

blood flow was 30% lower in cyclosporine-A treated renal allografts, although this did not reach significance. Calculated renal vascular resistance was twofold higher after transplantation and cyclosporine-A treatment than in control donor kidneys. Neither iminoethyl-lysine nor butylhexahydro-azepin-imine administration in addition to cyclosporine-A had any effect on mean arterial pressure or hematocrit compared to cyclosporine-A treatment only. Iminoethyl-lysine and in particular butylhexahydro-azepin-imine corrected the decrease in glomerular filtration rate of the renal graft toward glomerular filtration rate values found in control donors. Renal vascular resistance values were completely normalised by butylhexahydro-azepin-imine and were significantly lower than after treatment with cyclosporine-A alone. Renal blood flow values were not statistically different between the groups, but butylhexahydro-azepin-imine prevented any decrease from control donor levels.

3.3. Pathology

To examine the effect of iNOS inhibition on the pathologic features of renal allografts, we evaluated kidney weight, the extent of vascular, glomerular, interstitial and tubular lesions in cyclosporine-A alone compared to iNOS blocked renal transplants at 7 days post transplantation (Table 2; photographs in Figs. 1–3).

Weight of renal graft, spleen, and heart of the cyclosporine-A treated rats, with or without iNOS blockers, did not differ at 7 days after transplantation. No histological lesions were detected in the native kidneys of control rats. In renal allografts treated with low dose cyclosporine-A only, there were indications of acute cellular rejection with vascular, glomerular and tubulointerstitial lesions due to the residual inflammatory response. iNOS inhibition by iminoethyl-lysine or butylhexahydro-azepin-imine in addition to cyclosporine-A had no significant effects on total vascular and glomerular injury indices, although a clear tendency to less vascular lesions was found with a reduction in endothelial swelling, or thrombosis. Tubulointerstitial injury, however, was reduced significantly by both iminoethyl-lysine and butylhexahydro-azepin-imine. Renal cortex and outer medulla demonstrated decreased tubular swelling and tubular atrophy. Butylhexahydro-azepin-imine significantly reduced disruptions in proximal and distal tubular integrity, whereas iminoethyl-lysine treatment significantly reduced tubulitis (Fig. 1A,B,C). Interstitial injury, including amount and expansion of interstitial cell infiltrates, interstitial oedema, and occasional small haemorrhages declined after treatment with both iNOS blockers.

To determine the amount and expansion of inflammation, the number of monocytes per glomerulus and per tubulo-interstitial area was scored on ED1 stained kidney sections. In native kidneys of control rats, practically no adherent and infiltrating cells were found in glomeruli or around blood vessels. In the grafts treated with low doses of cyclosporine-A, with or without iNOS blockers, $ED1^+$-cells were the dominant infiltrating cell (Fig. 2A,B,C). Neither iminoethyl-lysine nor butylhexahydro-azepin-im-

Table 2
Vascular, glomerular and tubulointerstitial injury in renal allografts
Organ wet weight of transplanted kidney, spleen and heart of immunosuppressed control recipients (CyA, cyclosporine-A) and iNOS blocked recipients (CyA + L-NIL, cyclosporine-A + iminoethyl-lysine; CyA + BAI, cyclosporine-A + butylhexahydro-azepin-imine). Organ weights are expressed in gram per 100 g body weight. Semiquantitative histological analysis of allogenic renal transplants treated with cyclosporine-A alone, treated with cyclosporine-A + iminoethyl-lysine, or treated with cyclosporine-A + butylhexahydro-azepin-imine for 7 days after transplantation.
Data are mean ± S.E.M.; one way ANOVA on ranks (Dunn's method); T.I. = tubulointerstitial macrophages (per 1 high power field).

	CyA	CyA + L-NIL	CyA + BAI
n	7	7	7
body weight	29 ± 65	306 ± 11	308 ± 7
kidney weight	0.27 ± 0.01	0.27 ± 0.01	0.26 ± 0.01
spleen weight	0.21 ± 0.01	0.20 ± 0.01	0.22 ± 0.01
heart weight	0.31 ± 0.01	0.29 ± 0.01	0.32 ± 0.03
total vascular injury index	85.8 ± 12.0	69.7 ± 10.3	74.9 ± 8.3
glomerular lesion index	36.1 ± 8.5	33.1 ± 6.3	23.4 ± 2.8
macrophages/glomerulus	2.6 ± 0.5	2.1 ± 0.2	1.6 ± 0.2
tubulitis index	7.2 ± 1.0	3.4 ± 0.4[a]	4.9 ± 0.8
tubulo injury index	9.9 ± 1.9	6.9 ± 1.2	4.4 ± 1.0[a]
interstitial injury index	20.9 ± 1.7	14.6 ± 1.1[a]	15.2 ± 1.1[a]
T.I. macrophages	80 ± 18	66 ± 16	57 ± 7
nitrotyrosine index	8.5 ± 1.2	2.4 ± 0.5[a]	4.6 ± 0.8[a]

[a] $P < 0.05$ compared to treatment with cyclosporine-A alone.

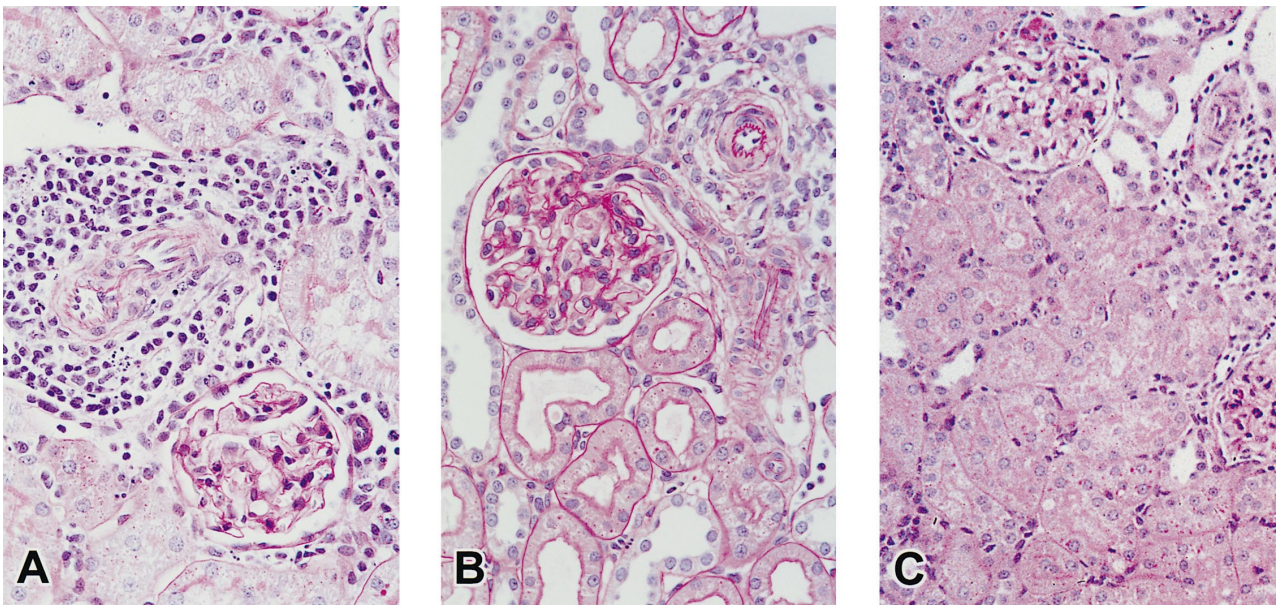

Fig. 1. (A) Allograft treated with low-dose cyclosporine-A, with normal arteriole and glomerulus, but severely injured and vacuolised tubules, with perivascular mononuclear cell infiltrate in oedematous areas (PAS staining; ×100). (B) Allograft treated with low-dose cyclosporine-A + iminoethyl-lysine, without vascular, glomerular and tubular damage. There is a slight rim of mononuclear cells around tubuli. This is significantly less than in (A) (PAS staining; ×100). (C) Allograft treated with low-dose cyclosporine-A + butylhexahydro-azepin-imine, without vascular and glomerular damage and only slight tubular lesions (PAS staining; ×100).

ine had a significant effect on the number of ED^+-cells in glomeruli or in interstitium (Table 2). However, the distribution of infiltrating cells was different. In cyclosporine-A treated grafts, the heavy leukocyte infiltration was intensified in large perivascular and periglomerular aggregates with predominantly $ED1^+$-cells and correlated with severe oedema formation and/or tubulointerstitial injury (Fig. 2A). In cyclosporine-A + iminoethyl-lysine treated grafts, the macrophages were scattered throughout the parenchyma (Fig. 2B). Cyclosporine-A + butylhexahydro-azepin-imine showed smaller perivascular cell infiltrates with slight extension to the interstitium (Fig. 2C).

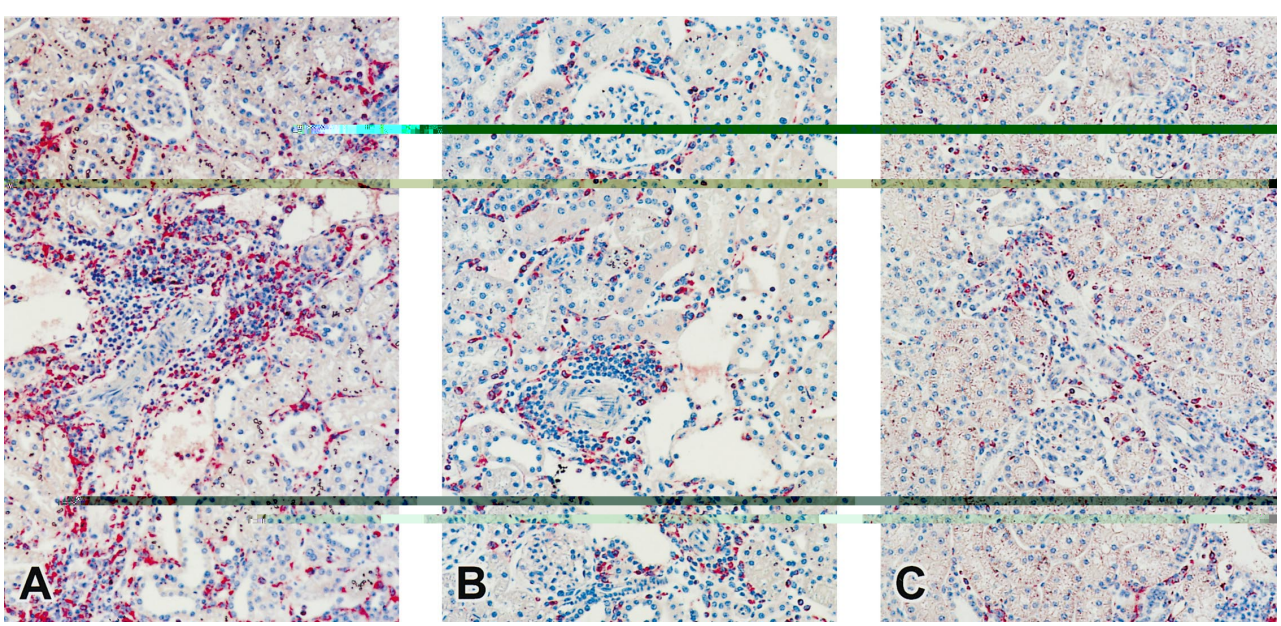

Fig. 2. (A) Allograft treated with low-dose cyclosporine-A demonstrated severe cortical diffuse mononuclear cell infiltration (ED1 staining; ×50). (B) Cyclosporine-A + iminoethyl-lysine treated allograft with cortically scattered infiltration of monocytes/macrophages (ED1 staining; ×50). (C) Cyclosporine-A + butylhexahydro-azepin-imine treated allograft, with perivascular and slight periglomerular focal infiltration of monocytes/macrophages (ED1 staining; ×50).

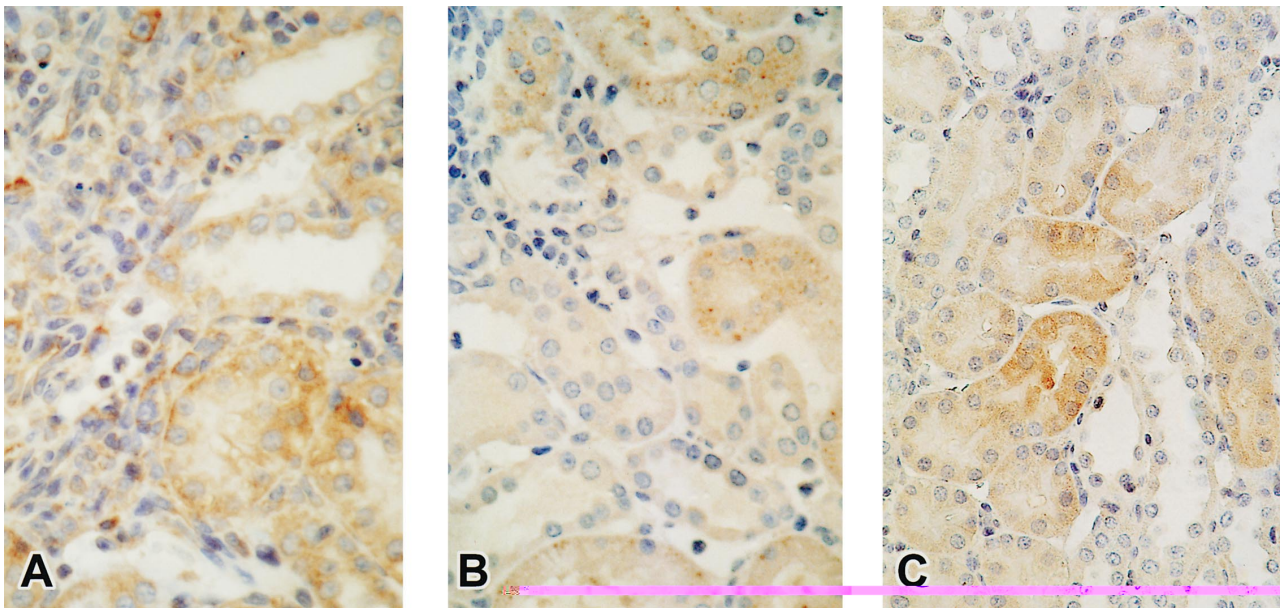

Fig. 3. (A) Nitrotyrosine in cyclosporine-A treated allograft, staining in proximal tubule and distal tubules and mononuclear cells (×200). (B) Nitrotyrosine in cyclosporine-A + iminoethyl-lysine treated allograft; no staining in distal tubules and mononuclear cells, significantly less positive staining in proximal tubules as compared to (A) (×200). (C) Nitrotyrosine in cyclosporine-A + butylhexahydro-azepin-imine treated allograft; no staining of mononuclear cells or distal tubules, significantly less positive staining in proximal tubules (×200).

Kidney grafts of recipients treated with cyclosporine-A alone showed significant nitrotyrosine staining detectable as cytoplasmic staining in the proximal and distal tubules of the inner cortex and outer medulla as well as on infiltrating mononuclear cells (Fig. 3A). In contrast, only pale tubular nitrotyrosine staining was detected in the allografts of iminoethyl-lysine + cyclosporine-A (Fig. 3B) and butylhexahydro-azepin-imine + cyclosporine-A treated recipients (Fig. 3C).

4. Discussion

Administration of the selective iNOS blockers, iminoethyl-lysine, and butylhexahydro-azepin-imine, to renal allografts treated with low-dose cyclosporine-A modulated the fall in renal function. Particularly, butylhexahydro-azepin-imine preserved glomerular filtration rate at near normal levels. Furthermore, both iminoethyl-lysine and butylhexahydro-azepin-imine had beneficial effects on tubulointerstitial morphology.

Iminoethyl-lysine and butylhexahydro-azepin-imine are considered to act as selective iNOS blockers as was shown by in vitro experiments. Mean arterial pressure did not change after iNOS blockade, which implies that these iNOS blockers in the dosage administered in vivo did not effectively block constitutive NOS of endothelium.

In the cyclosporine-A treated renal allograft, we observed a marked reduction of glomerular filtration rate and an increase — not reaching significance — of renal vascular resistance, as has been described previously during acute rejection (Munger et al., 1993). Cyclosporine-A + iminoethyl-lysine treatment tended to prevent the reduction in glomerular filtration rate, whereas cyclosporine-A + butylhexahydro-azepin-imine treatment completely restored both glomerular filtration rate and renal vascular resistance, implying a better recovery of acute renal failure due to surgery and alloimmunity. Possibly butylhexahydro-azepin-imine is a more effective agent than iminoethyl-lysine. Neither iminoethyl-lysine nor butylhexahydro-azepin-imine combined with cyclosporine-A caused differences in hematocrit and mean arterial pressure when compared to cyclosporine-A treatment only, indicating a direct effect of iminoethyl-lysine and butylhexahydro-azepin-imine on renal hemodynamics rather than a systemic effect. Some of the anti-inflammatory effects of iminoethyl-lysine have been attributed to inhibition of cyclo-oxygenase II (Salvemini et al., 1995). However, inhibition of cyclo-oxygenase II does not seem to have been of importance in the present study, because less synthesis of prostaglandins would have resulted in a decrease of glomerular filtration rate (Ter Wee et al., 1986) and renal blood flow (Ortiz et al., 1986).

The beneficial effects of iNOS inhibition on renal function suggest a role for NO produced by iNOS in contributing to progressive damage of glomerular and vascular structures. A tendency to the reduction in vascular damage and glomerular lesions was shown after iminoethyl-lysine and butylhexahydro-azepin-imine treatment. In contrast, sustained iNOS inhibition (i.e., 28 days of iminoethyl-lysine) in an aortic allograft model of chronic rejection (Shears et al., 1997), significantly increased intimal thick-

ening. A possible explanation could be that sustained iNOS induction mediates protection against myocyte proliferation and tissue scarring, by the antiproliferative actions of NO, while early iNOS induction, as in our model, mainly mediates a cytotoxic inflammatory response to the graft. Macrophages infiltrating into the graft during the initial inflammatory response and subsequent acute rejection are the major source of NO (Cattell et al., 1994; Goto et al., 1997) and O_2^-. Both NO and O_2^- can be produced via activated iNOS, especially in macrophages (MacMicking et al., 1997; Xia and Zweier, 1997). We postulated that active iNOS is an important macrophage effect or mechanism in graft rejection. In this study, the selective iNOS inhibitors, iminoethyl-lysine and butylhexahydro-azepin-imine, did not significantly decrease the number of macrophages, but the inflammatory pattern was different in that perivascular aggregation and the interstitial expansion of macrophages was limited. The latter was associated with less oedema and tubular atrophy, suggesting a restricted migration of macrophages towards primarily injured tubulointerstitial areas. Hence, blockade of iNOS in the early phase of transplant rejection may reduce reperfusion-mediated tubulointerstitial injury and the subsequent attraction of macrophages. Iminoethyl-lysine in fact was demonstrated to reduce nitrotyrosine formation and lipid peroxidation secondary to ischemia/reperfusion (Noiri et al., 1997). Moreover, our data on nitrotyrosine staining intensity suggest that iminoethyl-lysine and butylhexahydro-azepin-imine probably decreased the production of NO and/or O_2^-. Peroxynitrite, a strong stable oxidant (Beckman and Koppenol, 1996), has been demonstrated after ischemia/reperfusion (Noiri et al., 1997), in inflamed tissue (Heeringa et al., 1998), and in proximal tubular epithelium of rejecting human renal allografts (MacMillan-Crow et al., 1996). We demonstrated that iNOS blockade reduced tubulointerstitial nitrotyrosine staining, as well as damage. The tubulointerstitial damage possibly occurred in response to peroxynitrite formation. The decrease in macrophages infiltrated in the tubular epithelium after treatment with iminoethyl-lysine was possibly related to the decrease in nitrotyrosine staining.

In conclusion, this study shows that iNOS blockers, such as iminoethyl-lysine and butylhexahydro-azepin-imine, may have an additive effect on low-dose cyclosporine-A treatment, improving graft function and reducing early formation of tubulointerstitial lesions in the renal allograft. Independent of the therapeutic aspect, one should consider that these data strengthen the concept that NO generated by iNOS is significantly involved in acute rejection.

Acknowledgements

This study was supported by a grant from the Deutsche Forschungsgemeinschaft, Gr28/6-1 to H.-J.G., and by the Dutch Kidney Foundation.

The study has been presented in part at the 30th Annual Meeting of the American Society of Nephrology, San Antonio, TX, Nov. 2–5, 1997 and published in abstract form (J. Am. Soc. Nephrol., 8, 1997, A3116).

The excellent technical assistance of Mrs. C. Schmidt and the critical reading of the manuscript by H.J. Schuurman, Ph.D., are gratefully acknowledged.

References

Beckman, J.S., Koppenol, W.H., 1996. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am. J. Physiol. 21, C1424–C1437.

Buttery, L.D., Springall, D.R., Chester, A.H., Evans, T.J., Standfield, E.N., Parums, D.V., Yacoub, M.H., Polak, J.M., 1996. Inducible nitric oxide synthase is present within human atherosclerotic lesions and promotes the formation and activity of peroxynitrite. Lab. Invest. 75, 77–85.

Cattell, V., Smith, J., Jansen, A., Riveros-Moreno, V., Moncada, S., 1994. Localisation of inducible nitric oxide synthase in acute renal allograft rejection in the rat. Transplantation 58, 1399–1402.

De Graaf, J.C., Banga, J.D., Moncada, S., Palmer, R.M., De Groot, P.G., Sixma, J.J., 1992. Nitric oxide functions as an inhibitor of platelet adhesion under flow conditions. Circulation 85, 2284–2290.

Fisher, B., Lee, S., 1965. Microvascular surgical techniques in research with special reference to renal transplantation in the rat. Surgery 58, 904–914.

Floege, J., Gröne, H.-J., 1995. Progression of renal failure: what is the role of cytokines? Nephrol. Dial. Transplant. 10, 1575–1586.

Fransen, R., Koomans, H.A., 1995. Adenosine and renal sodium handling: direct natriuresis and renal nerve-mediated antinatriuresis. J. Am. Soc. Nephrol. 6, 1491–1497.

Gauthier, T.W., Scalia, R., Murohara, T., Guo, J.P., Lefer, A.M., 1995. Nitric oxide protects against leukocyte-endothelium interactions in the early stages of hypercholesterolemia. Arterioscler., Thromb., Vasc. Biol. 15, 1652–1659.

Goto, M., Yamaguchi, Y., Ichiguchi, O., Miyanari, N., Akizuki, E., Matsumura, F., Matsuda, T., Mori, K., Ogawa, M., 1997. Phenotype and localization of macrophages expressing inducible nitric oxide synthase in rat hepatic allograft rejection. Transplantation 64, 303–310.

Heeringa, P., van Goor, H., Moshage, H., Klok, P.A., Huitema, M.G., de Jager, A., Schep, A.J., Kallenberg, C.G., 1998. Expression of iNOS, eNOS, and peroxynitrite-modified proteins in experimental anti-myeloperoxidase associated crescentic glomerulonephritis. Kidney Int. 53, 382–393.

Langrehr, J.M., Murase, N., Markus, P.M., Cai, X., Neuhaus, P., Schraut, W., Simmons, R.L., Hoffman, R.A., 1992a. Nitric oxide production in host-versus-graft and graft-versus-host reactions in the rat. J. Clin. Invest. 90, 679–683.

Langrehr, J.M., Simmons, R.L., Hoffman, R.A., 1992b. Graft-infiltrating cell nitric oxide production is stimulated by TNF-alpha. Transplant. Proc. 24, 2893.

Langrehr, J.M., White, D.A., Hoffman, R.A., Simmons, R.L., 1993. Macrophages produce nitric oxide at allograft sites. Ann. Surg. 218, 159–166.

MacMicking, J., Xie, Q.W., Nathan, C., 1997. Nitric oxide and macrophage function. Annu. Rev. Immunol. 15, 323–350.

MacMillan-Crow, L.A., Crow, J.P., Kerby, J.D., Beckman, J.S., Thompson, J.A., 1996. Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. Proc. Natl. Acad. Sci. USA 93, 11853–11858.

Mclay, J.S., Chatterjee, P., Nicolson, A.G., Jardine, A.G., Mckay, N.G., Ralston, S.H., Grabowski, P., Haites, N.E., Macleod, A.M., Hawksworth, G.M., 1994. Nitric oxide production by human proximal

tubular cells: a novel immunomodulatory mechanism? Kidney Int. 46, 1043–1049.

Moore, W.M., Webber, R.K., Jerome, G.M., Tjoeng, F.S., Misko, T.P., Currie, M.G., 1994. L-*N*6-(1-iminoethyl)lysine: a selective inhibitor of inducible nitric oxide synthase. J. Med. Chem. 37, 3886–3888.

Munger, K.A., Coffman, T.M., Griffiths, R.C., Fogo, A., Badr, K.F., 1993. The effects of surgery and acute rejection on glomerular hemodynamics in the transplanted rat kidney. Transplantation 55, 1219–1224.

Noiri, E., Nakao, A., Uchida, K., Kimura, S., Goligorsky, M.S., 1997. Selective inducible NOS inhibition ameliorates lipid peroxidation in rat ischemic acute renal failure. J. Am. Soc. Nephrol. 8, A2760, (Abstract).

Ortiz, M.C., Atucha, N.M., Lahera, V., Vargas, F., Quesada, T., Garcia-Estan, J., 1986. Importance of nitric oxide and prostraglandins in the control of rat renal papillary blood flow. Hypertension 27, 377–381, (3Pt 1).

Salvemini, D., Manning, P.T., Zweifel, B.S., Seibert, K., Connor, J., Currie, M.G., Needleman, P., Masferrer, J.L., 1995. Dual inhibition of nitric oxide and prostaglandin production contributes to the anti-inflammatory properties of nitric oxide synthase inhibitors. J. Clin. Invest. 96, 301–308.

Salvemini, D., Wang, Z.Q., Wyatt, P.S., Bourdon, D.M., Marino, M.H., Manning, P.T., Currie, M.G., 1996. Nitric oxide: a key mediator in the early and late phase of carrageenan-induced rat paw inflammation. Br. J. Pharmacol. 118, 829–838.

Schwartz, D., Mendonca, M., Schwartz, I., Xia, Y., Satriano, J., Wilson, C.B., Blantz, R.C., 1997. Inhibition of constitutive nitric oxide synthase (NOS) by nitric oxide generated by inducible NOS after lipopolysaccharide administration provokes renal dysfunction in rats. J. Clin. Invest. 100, 439–448.

Shears, L.L., Kawaharada, N., Tzeng, E., Billiar, T.R., Watkins, S.C., Kovesdi, I., Lizonova, A., Pham, S.M., 1997. Inducible nitric oxide synthase suppresses the development of allograft arteriosclerosis. J. Clin. Invest. 100, 2035–2042.

Stojanovic, T., Gröne, H.-J., Gieseler, R.K., Klanke, B., Schlemminger, R., Tsikas, D., Gröne, E.F., 1996. Enhanced renal allograft rejection by inhibitors of nitric oxide synthase: a nonimmunologic influence on alloreactivity. Lab. Invest. 74, 496–512.

Szabolcs, M.J., Ravalli, S., Minanov, O., Sciacca, R.R., Michler, R.E., Cannon, P.J., 1998. Apoptosis and increased expression of inducible nitric oxide synthase in human allograft rejection. Transplantation 65, 804–812.

Takada, M., Nadeau, K.C., Shaw, G.D., Marquette, K.A., Tilney, N.L., 1997. The cytokine-adhesion molecule cascade in ischemia/reperfusion injury of the rat kidney. Inhibition by a soluble P-selectin ligand. J. Clin. Invest. 99, 2682–2690.

Ter Wee, P.M., Rosman, J.B., Van der Geest, S., Sluiter, W.J., Donker, A.J., 1986. Renal hemodynamics during separate and combined infusion of amino acids and dopamine. Kidney Int. 29, 870–874.

Wever, R.M.F., Van Dam, T., Van Rijn, H.J., De Groot, F., Rabelink, T.J., 1997. Tetrahydrobiopterin regulates superoxide and nitric oxide generation by recombinant endothelial nitric oxide synthase. Biochem. Biophys. Res. Commun. 237, 340–344.

Winlaw, D.S., Schyvens, C.G., Smythe, G.A., Du, Z.Y., Rainer, S.P., Lord, R.S., Spratt, P.M., Macdonald, P.S., 1995. Selective inhibition of nitric oxide production during cardiac allograft rejection causes a small increase in graft survival. Transplantation 60, 77–82.

Wolff, D.J., Lubeskie, A., Gauld, D.S., Neulander, M.J., 1998. Inactivation of nitric oxide synthases and cellular nitric oxide formation by *N*6-iminoethyl-L-lysine and *N*5-iminoethyl-L-ornithine. Eur. J. Pharmacol. 350, 325–334.

Worrall, N.K., Boasquevisque, C.H., Misko, T.P., Sullivan, P.M., Ferguson, T.B., Patterson, G.A., 1997. Inducible nitric oxide synthase is expressed during experimental acute lung allograft rejection. J. Heart Lung Transplant. 16, 334–339.

Worrall, N.K., Lazenby, W.D., Misko, T.P., Lin, T.S., Rodi, C.P., Manning, P.T., Tilton, R.G., Williamson, J.R., Ferguson, T.B., 1995. Modulation of in vivo alloreactivity by inhibition of inducible nitric oxide synthase. J. Exp. Med. 181, 63–70.

Worrall, N.K., Misko, T.P., Sullivan, P.M., Hui, J.J., Ferguson, T.B., 1996. Inhibition of inducible nitric oxide synthase attenuates established acute cardiac allograft rejection. Ann. Thorac. Surg. 62, 378–385.

Xia, Y., Zweier, J.L., 1997. Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. Proc. Natl. Acad. Sci. USA 94, 6954–6958.