Transpl Int (1996) 9: 137–140

ORIGINAL ARTICLE

Uwe W. Heemann
Stefan G. Tullius
Christof Schmid
Thomas Philipp
Nicholas L. Tilney

Infection-associated cellular activation accelerates chronic renal allograft rejection in rats

Received: 9 May 1995
Received after revision: 11 August 1995
Accepted: 26 September 1995

U.W. Heemann (✉) · T. Philipp
Department of Nephrology IG1–9,
University Hospital Essen,
Hufelandstraße 55,
D-45122 Essen, Germany
Fax: + 49 201 325 456

S.G. Tullius · C. Schmid · N.L. Tilney
Surgical Research Laboratory,
Harvard Medical School
Department of Surgery,
Brigham and Women's Hospital,
Boston, MA 02115, USA

Abstract The etiology of chronic rejection is unknown, although acute rejection, viral infection, and initial graft ischemia have been implicated. To test the effects of infections on the process of chronic rejection, we simulated bacterial infection by the administration of the endotoxin lipopolysaccharide (LPS), a potent activator of various cell types in an established rat model of chronic rejection. Lewis recipients of Fisher 344 kidneys were treated with a single dose of LPS or vehicle 8 weeks following transplantation and grafts were examined at various time points. In the chronically rejecting controls, leukocytic infiltration and the expression of cytokines peaked at 16 weeks. In LPS-treated hosts, leukocyte infiltration and cytokine expression peaked at 12 weeks. By 16 weeks, glomeruli in LPS-treated recipients had become far more sclerotic than those in controls, mimicking the changes observed in controls at 24 weeks. We conclude that infections may play an important role in the development of chronic rejection.

Key words Kidney transplantation, chronic rejection, rat · Kidney transplantation, lipopolysaccaride, rat · Rejection, chronic, lipopolysaccharide, rat kidney

Introduction

Despite progressive improvements in patient care and immunosuppression, renal transplantation has not realized its potential as a longlasting treatment for end-stage renal failure. The rate of attrition over the long term has remained constant for two decades; the majority of grafts are lost in the poorly defined process of chronic rejection [6]. Although its etiology is unknown, a number of risk factors such as early acute rejection episodes, initial ischemia, and viral infection have been associated with this enigmatic condition [2, 3, 9]. The present experiments examine the influence of infection-associated cellular activation on the development of chronic rejection of kidney allografts in a well-established model in rats [7, 10]. To standardize the effects of cellular activation and to prevent direct bacterial interactions and antibiotic therapy, we simulated infection by the administration of lipopolysaccharide (LPS), an endotoxin and a potent and well-known cell activator [16].

Methods

Transplantation

Naive, male, inbred rats, weighing 200–250 g and obtained from Harlan Sprague-Dawley (Indianapolis, Ind., USA), were used throughout the experiments. Lewis (LEW, $RT1^l$) rats acted as graft recipients, Fisher (F344, $RT1^{lv1}$) rats as donors.

The left donor kidney was isolated, harvested, cooled, and positioned orthotopically in the host whose left renal vessels had been dissected free and clamped and whose native kidney had been removed. The donor and recipient renal artery, vein, and ureter were then anastomosed end-to-end with 10–0 prolene sutures. No ureteral stent was used. All animals were treated briefly with low-dose cyclosporin (1.5 mg/kg per day for 10 days) to reverse an initial rejection episode [7 ,10]. The remaining right kidney of the recipient animal was removed on day 10, at which time the trans-

planted kidney was checked for surgical damage. Rats with any overt signs of unsuccessful operation were discarded.

During a period of functional and morphological stability (8 weeks), when the recipients were sustained well by their transplants, animals received a nonlethal dose (2 mg in 1 ml NaCl) of LPS i.p. (Sigma, St. Louis, Mo., USA). Control rats were treated in parallel with 1 ml of NaCl.

Proteinuria

Urine samples (24 h) were collected every 4 weeks. Protein excretion was determined by precipitation with 3 % sulfosalicylic acid. Turbidity was then assessed by absorbance at a wavelength of 595 nm using a Coleman Junior II spectrophotometer [8].

Harvesting

Transplanted kidneys from LPS-treated animals were harvested at 12, 16, and 24 weeks and from non-LPS-treated controls at 8 (before treatment), 12, 16, and 24 weeks after transplantation. Eight animals were included in each group/time point. Only kidneys without complications of grafting (pyelonephritis or hydronephrosis) were included in the study.

Representative portions of all renal tissues were snap-frozen in liquid nitrogen and stored at –70 °C for immunohistology or fixed in buffered formalin (10 %) for morphology.

Histology

Formalin-fixed sections were stained with hematoxylin/eosin for routine histology (infiltration, tubular atrophy) and with periodic acid-Schiff (PAS) to evaluate the extent of glomerulo- and arteriosclerosis. At least 200 glomeruli were counted in each kidney and the proportion of sclerosed to total glomeruli were expressed as a percentage.

Antibodies

Various monoclonal antibodies (mAbs) were used for immunohistology. A mAb against ICAM-1 (1A29) was provided by Prof. M. Miyasaka (Tokyo, Japan) [14]. Those against lymphocytes (CD5–OX-19, predominantly T cells) and monocyte/macrophages (ED-1) were obtained from Bioproducts for Science (Indianapolis, Ind., USA), those against laminin and fibronectin from Gibco (Grand Island, N.Y., USA) and those against TNFα, TGFβ, and PDGF-BB, from Genzyme (Boston, Mass., USA).

Immunohistology

Cryostat sections of frozen tissues were stained individually with appropriate mAbs using alkaline-phosphatase-anti-alkaline-phosphatase, immunoperoxidase (laminin, fibronectin). Stained cells were then counted on an ocular grid and expressed as cells per field of view (c/FV; × 600, > 30 fields counted/specimen, two to three specimens/kidney). The intensity of tissue staining of ICAM-1, cytokines and growth factors was evaluated on a scale of 0–4, where 0 represented no staining, 1 minimal staining, and 4 intense staining.

Statistics

Data are presented as mean ± standard error of mean. Differences between groups were assessed using an ANOVA and Scheffé's *t*-test, as appropriate [15].

Results

Functional and morphological changes

The F344 → Lew rat strain combination has provided a reproducible model of chronic kidney allograft rejection [7, 10]. Proteinuria developed in control recipients by 12 weeks, increasing progressively thereafter and correlating with worsening glomerular sclerosis and intimal proliferation of arteries and arterioles; by 24 weeks, generalized fibrosis had become obvious. Administration of LPS accelerated the development of these morphological and functional chronic changes (Table 1). LPS was administered at 8 weeks, a period of quiescence in the non-LPS-treated recipients. By 16 weeks, proteinuria in LPS treated rats had increased to levels significantly exceeding those of untreated controls (79 mg/24 h vs 49 mg/24 h, $P < 0.05$). At the same time, 35 % of all allograft glomeruli in LPS-treated rats had become sclerotic compared to 15 % in controls ($P < 0.01$; Table 1), while the degree of interstitial fibrosis was considerably higher. Between 16 and 24 weeks, four LPS-treated animals died due to kidney failure, while none of the controls was lost during the period observed.

Immunohistology

Immunohistologically, the numbers of CD5 + lymphocytes remained consistently low (< 25 c/FV) in the chronically rejecting allografts in treated and untreated hosts over the whole period observed. Infiltrating ED1 + macrophages, present in small numbers throughout the allograft tissue until 12 weeks, increased dramatically at 16 weeks (> 70 c/FV), declining to baseline by 24 weeks (Table 1). The increased presence of this cell population between 12 and 16 weeks in and around vessels and inside glomeruli occurred in parallel with a dramatic upregulation of adhesion molecules, cytokines, and growth factors (Table 2).

At 8 weeks, few areas of leukocyte infiltration were detected in the grafts, and the expression of adhesion molecules, cytokines, and growth factors was focused on these areas. ICAM-1, while detectable throughout the allografts on vascular endothelial cells, was expressed with a low intensity (1–2 +).

At 12 weeks, leukocyte infiltration and cytokine expression had increased only slightly. TNFα and ICAM-

Table 1 The degree of kidney damage increased over time in both controls and LPS-treated kidneys. In LPS-treated rats, however, the process developed in an accelerated fashion, with findings at week 12 resembling changes observed in controls at 16 weeks. The pattern observed in LPS-treated kidneys at 16 weeks was indistinguishable from that seen in controls at week 24. All values are given as mean ± SEM

	Untreated	Control allografts			LPS-treated allografts		
Weeks	8	12	16	24	12	16	24
n	8	8	8	8	8	8	4
Proteinuria (mg/24 h)	18 ± 8	40 ± 2	49 ± 6	69 ± 8	60 ± 6*	79 ± 5**	89 ± 7**
Glomerulosclerosis (%)	7 ± 5	11 ± 6	15 ± 6	32 ± 5	15 ± 5	35 ± 3**	67 ± 11**
Macrophages c/F	30 ± 6	30 ± 10	> 70	41 ± 9	> 70*	35 ± 7**	37 ± 6

* $P < 0.05$, ** $P < 0.01$ for treated compared to untreated allografts as the same time

Table 2 The pattern of cytokine expression in LPS-treated animals at 12 weeks was undistinguishable from that of untreated controls at 16 weeks. Values are given as the degree of intensity ± standard deviation

	Untreated	Control allografts			LPS-treated allografts		
Weeks	8	12	16	24	12	16	24
n	8	8	8	8	8	8	4
ICAM-1	1.3 ± 0.1	1.8 ± 0.2	3.7 ± 0.2	1.5 ± 0.3	3.6 ± 0.1	1.7 ± 0.3	1.8 ± 0.4
TNFα	0.9 ± 0.2	1.9 ± 0.1	3.6 ± 0.2	1.7 ± 0.3	3.2 ± 0.1	2.1 ± 0.3	1.4 ± 0.4
TGFβ	Rare	Rare	20 % of infiltrating leukocytes	20 % of infiltrating leukocytes	20 % of infiltrating leukocytes	20 % of infiltrating leukocytes	20 % of infiltrating leukocytes
PDGF-BB	Rare	Rare	20 % of infiltrating leukocytes	20 % of infiltrating leukocytes	20 % of infiltrating leukocytes	20 % of infiltrating leukocytes	20 % of infiltrating leukocytes
Laminin	1.5 ± 0.3	3.7 ± 0.1	3.7 ± 0.1	3.6 ± 0.1	3.6 ± 0.1	3.4 ± 0.1	3.9 ± 0.2
Fibronectin	1.6 ± 0.2	3.6 ± 0.2	3.7 ± 0.2	3.4 ± 0.2	3.5 ± 0.2	3.6 ± 0.2	3.8 ± 0.2

1-positive cells were more pronounced than at 8 weeks, but the overall expression was limited to the small number of areas where leukocytes had infiltrated, particularly around vessels.

At 16 weeks, dense infiltrates of leukocytes were accompanied by a dramatically increased expression of ICAM-1 on tubular epithelial and vascular endothelial cells, as well as on infiltrating leukocytes (3–4 + vs 1–2 + at 8 weeks). In areas of leukocyte infiltration, around 20 % of leukocytes labeled for TNFα, TGFβ, and PDGF-BB were detectable and most intense at areas of leukocyte infiltrations around vessels and glomeruli.Laminin expression was markedly increased on glomeruli developing sclerosis after 12 weeks (4 + vs 1–2 at 8 weeks), while fibronectin upregulation (3–4 +) between 8 and 12 weeks preceded macrophage infiltration slightly, particularly throughout the interstitium.

By 24 weeks, leukocyte infiltration and expression of ICAM-1, TNFα, TGFβ, and PDGF-BB was reduced, and fibrosis and sclerosis predominated.

These immunohistological changes were dramatized by the administration of LPS. At 12 weeks, macrophage infiltration (> 70 c/FV) was markedly higher than in untreated animals (30 ± 10 c/FV, $P < 0.05$), appearing in numbers similar to those observed in these control recipients at 16 weeks. At 12 weeks, the pattern of expression of PDGF-BB, TNFα, TGFβ, and other molecules including laminin, fibronectin, and ICAM-1 was indistinguishable from that observed in control allografts at 16 weeks. With the exception of laminin and fibronectin, the expression of these molecules decreased in LPS-treated animals as compared to LPS-treated animals at week 12, thus resembling the pattern observed in control animals at 24 weeks. At 24 weeks, the intensity of PDGF-BB, TGFβ, and TNFα was minimal, as were the areas of leukocyte infiltration in LPS-treated kidneys. Fibronectin and laminin expression was vast.

In summary, manifestations of chronic rejection evolved more rapidly in LPS-treated animals bearing long-time allografts than in untreated controls.

Discussion

The etiology of chronic rejection is unknown, probably multifaceted, and has been associated with both early alloantigen-dependent injury, such as acute rejection episodes, and nonalloantigen-dependent mechanisms, such as kidney mass [12], ischemia, reperfusion, and CMV infection [2, 3, 9]. The present study was designed

to assess the effects of infections, as a manifestation of such nonalloantigen-dependent mechanisms, on the course of chronic rejection. To simulate these events we administered LPS, an endotoxin with a high potential for cellular activation, at 8 weeks, a period of relative functional and morphological quiescence in this model. By 12 weeks (4 weeks later), important functional, morphological, and immunohistological changes had evolved within the grafts, similar to those occurring later in grafts of untreated animals. Indeed, it was not until 16 weeks that non-LPS-treated recipients developed comparable proteinuria, intense macrophage infiltration of graft substance, and significant cytokine upregulation [10].

LPS is known for its potential to stimulate various cell types, endothelial cells amongst them. Its effects include the induction of proliferation but also the upregulation of adhesion molecules, MHC class I and MHC class II. The expression of these molecules and their importance in transplant models is well documented [1, 11, 13]. In isolated erythrocyte-perfused rat kidneys, LPS stimulated the production of monocyte chemoattractant protein-1 (MAP-1), interleukin-1 beta (IL-1 beta), and TNFα [16]. Our results fit those observations as we also observed an elevated level of TNFα expression. However, the mechanism of action remains dubious. LPS not only activates endothelial cells but also activates macrophages. As we observed the highest intensity of growth factor expression in areas of macrophage infiltration, it is possible that macrophage activation is the predominant factor. On the other hand, LPS is a specific mitogen for B cells [5]. In these experiments we did not look for antibody deposition, but in previous studies antibody labeling was detectable to some degree, indicating that the deposition of IgG is a possible factor in the etiology of chronic rejection [10]. While unlikely to play a major role, antibody deposition may induce complement activation and/or neutrophil infiltration, thereby damaging the kidney.

Previous studies have indicated the importance of factors such as decreased functioning kidney mass, CMV infections, and sex and age of donor [4, 9] for the development of chronic rejection. The earlier onset of chronic rejection in our model following the application of LPS demonstrates the importance of these nonallogenic factors in the etiology of this process. LPS may accelerate the chronic process by activation of endothelial cells and/or infiltrating leukocytes. Most likely the effects of LPS are due to additional kidney damage triggered by a combination of an increased infiltration of leukocytes, the activation of macrophages and, possibly, the activation of the complement system.

Acknowledgements This work was supported by USPHS Grant 9 R01 DK 46190–21. U. Heemann, S. Tullius and C. Schmid are recipients of a Research Fellowship award from the Deutsche Forschungsgemeinschaft (DFG) (He-1906/3–1, Tu-63/1–1, Schm-1029/1-1).

References

1. Azuma H, Heemann UW, Tullius SG, Tilney NL (1994) Cytokines and adhesion molecules in chronic rejection. Clin Transplant 8: 168
2. Basadonna GP, Matas AJ, Gillingham KJ, Payne WD, Dunn DL, Sutherland DER, Gores PF, Grüssner RWG, Arrazola L, Najarian JS (1993) Relationship between early vs. late acute rejection and onset of chronic rejection in kidney transplantation. Transplant Proc 25: 910
3. Brazy PC, Pirsch JD, Belzer FO (1992) Factors affecting renal allograft function in long-term recipients. Am J Kidney Dis 19: 558
4. Brenner BM, Milford EL (1993) Nephron underdosing: a programmed cause of chronic renal allograft failure. Am J Kidney Dis 21: 66
5. Bucala R (1992) Polyclonal activation of B lymphocytes by lipopolysaccharide requires macrophage-derived interleukin-1. Immunology 77: 477–482
6. Cook D S, Terasaki PI (1989) Renal transplantation on the American continent. In: Brent L, Salks RA (eds) Organ transplantation: current clinical and immunological concepts. Bantham Tindall, London, pp 195–199
7. Diamond JR, Tilney NL, Frye J, Ding G, McElroy J, Pesek-Diamond I, Yang H (1992) Progressive albuminuria and glomerulosclerosis in a rat model of chronic renal allograft rejection. Transplantation 54: 710
8. Freeman JA, Beeler MF (1983) Laboratory medicine/urinanalysis and medical microscopy. Lea & Febiger, Philadelphia, p 148
9. Grattan MT, Moreno-Cabral CE, Starnes VA (1989) Cytomegalovirus infection is associated with cardiac allograft rejection and atherosclerosis. JAMA 261: 3561
10. Hancock WW, Whitley WD, Tullius SG, Heemann UW, Wasowska B, Baldwin WM III, Tilney NL (1993) Cytokines, adhesion molecules and the pathogenesis of chronic rejection of rat renal allografts. Transplantation 56: 643
11. Heemann UW, Tullius SG, Tilney NL (1992) First host lymphocyte contact with acutely rejecting kidneys in rats depends on adhesion molecules and not on prior sensitization. Surg Forum 18: 429
12. Heemann UW, Tullius SG, Azuma H, Schmid C, Tilney NL (1994) Contribution of reduced functioning kidney mass to chronic renal allograft dysfunction in rats. Transplantation 58: 1317–1322
13. Heemann UW, Tullius SG, Tamatami T, Miyasaka M, Milford E, Tilney NL (1994) Infiltration patterns of macrophages and lymphocytes in chronically rejecting rat kidney allografts. Transpl Int 7: 349–355
14. Tamatami T, Kotani M, Miyasaka M (1991) Characterization of the rat leukocyte integrin CD11/CD18 by the use of LFA-1 subunit-specific monoclonal antibodies. Eur J Immunol 21: 627–633
15. Wallenstein S, Zucker L, Fleiss JL (1980) Some statistical methods useful in circulation research. Circ Res 47: 1–9
16. Xia Y, Feng L, Yoshimura T, Wilson CB (1993) LPS-induced MCP-1, IL-1 beta, and TNF-alpha mRNA expression in isolated erythrocyte-perfused rat kidney. Am J Physiol 264: F774