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Transplantation of a single kidney *per se* does not lead to late graft dysfunction

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Abstract In unraveling the pathogenesis of chronic transplant dysfunction (CTD), non-alloantigen specific factors, as ischemia/reperfusion and renal mass have been suggested to play a role in the process. The aim of the present study was to investigate the effect of the transplantation procedure *per se* on the development of CTD in a syngeneic kidney transplant model in the rat. Kidney transplantation was performed with the BN rat as donor and recipient, the recipient kidneys having been removed. Unilaterally nephrectomized (UNx) and native BN rats served as controls. Renal function was determined monthly (proteinuria and glomerular filtration rate/100 g body weight; GFR). The follow-up period was until 52 weeks post-transplantation. Histomorphological analysis of CTD according to the BANFF criteria was carried out. Immunohistochemical staining was performed to identify infiltrating cells (CD4, CD8, and ED1) and the expression of MHC class II and ICAM-1. Isografts had a minor, constant proteinuria during follow-up, which did not differ from that of UNx: 27 ± 10 vs. 29 ± 2 mg/24 h at week 52. Unilateral nephrectomy led to a significant reduction of the GFR, which was about 80% of that of native rats. The GFR of isografts did not differ from that of UNx rats. Histomorphology of renal isografts was comparable to UNx and native kidneys; some glomerulopathy and tubular atrophy leading to a total BANFF-score of 2.6 ± 0.5. In native BN kidneys, few $CD4^+$ cells and $ED\text{-}1^+$macrophages (mΦ) were found; MHC class II was constitutively expressed on the proximal tubules and ICAM-1 on the glomeruli and peritubular capillaries. UNx-kidneys showed a similar pattern. Isografts had significantly more $CD4^+$cells and MΦ, mainly localized in the glomeruli, and a more intense ICAM-1 expression in the glomeruli and interstitium. Transplantation of one kidney in itself does not lead to CTD.

Keywords Transplantation · Kidney · Ischemia · Renal Mass · Chronic Transplant Dysfunction

Abbreviations *BW* Body weight · *BP* Blood pressure · *CTD* Chronic transplant dysfunction · *GFR* Glomeraular filtration rate · *UNx* Unilaterally nephrectomized

Introduction

Despite progressive improvements in the early success rate of clinical organ transplantation, the annual rate of graft loss after the first year has not improved significantly over the last 2 decades. The half-life of cadaveric renal allografts remains consistent at 8–9 years [8]. Late failure of renal allografts is largely attributable to

the poorly understood process of chronic transplant dysfunction (CTD). The cardinal histopathological characteristic of CTD is intimal hyperplasia [11]; the chronic dysfunctioning kidney graft also shows less specific features, such as glomerular sclerosis, tubular atrophy and interstitial fibrosis. The mechanisms leading to CTD are largely unknown [9, 14]. Clinical data indicate that the host alloimmune response plays an important role in the development of CTD: Acute rejection is the most consistent risk factor for CTD [7]. Nonetheless, there are indications that alloantigen-independent factors can cause lesions resembling those seen in chronically rejected allografts. For instance, renal ischemia in unilaterally nephrectomized rats can cause late histomorphological lesions mirroring those seen in kidney allografts with CTD [10, 13]. In syngeneic kidney transplants, Tullius *et al* demonstrated late damage to the same extent as observed in ischemic non-transplant kidneys [13]. Others, however, did not see functional or morphological changes in renal isografts [5, 16]. The pathway from such an early injury to the late morphological changes is unclear. Since reduction of nephron number in non-transplant experiments can cause glomerular and vascular changes, thus resembling CTD [3], it has been suggested that ischemic injury leads to a decrease of nephron number. To further unravel the etiology and pathophysiology of CTD, the aim of the present study was to investigate the effect of the transplant procedure on CTD in the rat kidney. To rule out alloimmune mediated effects on CTD, syngeneic transplants were studied.

Materials and methods

Animals

Young, adult male, inbred Brown Norway rats (BN; RT-1^n) weighing 225–250 g and 10–12 weeks old were purchased from Harlan, Austerlitz, The Netherlands. All animals were kept under standard conditions and given access to standard commercial rat chow (AM II; Hope Farms, Woerden, The Netherlands) and tap water acidified to pH 3, *ad libitum*. The experimental protocols were approved by the committee on animal research of the Erasmus University, Rotterdam.

Kidney transplantation

Kidney transplantation was performed using a modification of the technique described by Fisher and Lee [2]. The animals were anesthetized with ether. After an intravenous injection of heparin (100 IU), the left donor kidney was flushed in situ via the aorta with 5 ml University of Wisconsin (UW) solution at 4 °C at a rate of 2 ml /min. The kidney was excised and stored in UW solution (4 °C) for about 6 min prior to implantation. In the recipient, the kidney graft was transplanted heterotopically; donor renal artery and vein were anastomosed end-to side to recipient aorta and vena cava, respectively, using continuous 9–0 prolene sutures (B.Braun, Melsungen, Germany). During surgery, the graft was wrapped in gauze moisturized with 4 °C phosphate buffered saline (PBS). The perioperative ischemia time was 30 min. After revascularization, the ureter was anastomosed end-to-end to the distal third part of the recipient's ureter with 4 single 10-0 prolene sutures, without use of a stent. The left native kidney was removed at time of transplantation, while the contralateral one was excised 3 weeks later.

Experimental groups

BN isografts were followed up until 52 weeks ($n = 5$) after transplantation. Control groups were age-matched, unilaterally nephrectomized (UNx) rats ($n = 8$), and native non-nephrectomized rats $n = 5$). Functional, morphological, and immunohistological evaluations were performed after engraftment.

Functional measurements

Urine was collected monthly by placing the rats individually for 24 h in metabolic cages. Protein excretion was measured colorimetrically by addition of pyrogallol red [15]. The glomerular filtration rate (GFR) per 100 g body weight (BW) was based on the clearance of creatinine. Serum and urinary creatinine was determined using the Jaffé method without deproteinization.

Before the rats were killed, their diastolic and systolic blood pressure (BP) was measured intra-arterially. After anesthetizing the rat, the right carotid artery was dissected, a pressure-probe (Baxter, United Kingdom) was inserted into the direction of the aortic arch, and the BP was recorded continuously for 5 min.

Histology

At 52 weeks post-transplantation, kidneys were harvested and weighed. They were fixed by immersion for 48 h in a 3.6% buffered formaldehyde solution after longitudinal bisection, and embedded in paraffin. Sections (3 μm) were stained with hematoxylin/eosin, periodic acid Schiff (PAS) and evaluated for chronic transplant dysfunction according to the BANFF-criteria [12] by 2 independent investigators. Briefly, glomerulopathy, interstitial fibrosis, tubular atrophy, and intimal thickening were separately determined on a score ranging from 0 = normal, 1 = up to 25% affected, 2 = moderateley, affected 25–50%, and 3 = more than 50% changes. The total BANFF score is the sum of the 4 individual scores.

Immunohistology

Representative portions of all kidneys were stained on 5μm cryostat sections by a three-layer immunoperoxidase technique. After fixation with acetone for 10 min, tissues were dehydrated through graded alcohols to block endogenous peroxidase activity by incubation for 10 min in methanol/ 0.03% H_2O_2. After rehydration, the non-specific binding was blocked by preincubation with 10% Normal Rabbit serum (Dako, Copenhagen, Denmark), in PBS/Bovine serum Albumin 5%. This was followed by 1 h of incubation with primary monoclonal antibodies (Serotec, Oxford, United Kingdom) for identification of CD45$^+$ leucocytes (OX-1), CD4$^+$ cells (W3/25), CD8$^+$ cells (OX-8), monocytes/macrophages (ED-1), major histocompatibility complex (MHC) class II antigens (OX-6), and intercellular adhesion molecule 1 (ICAM-1). After

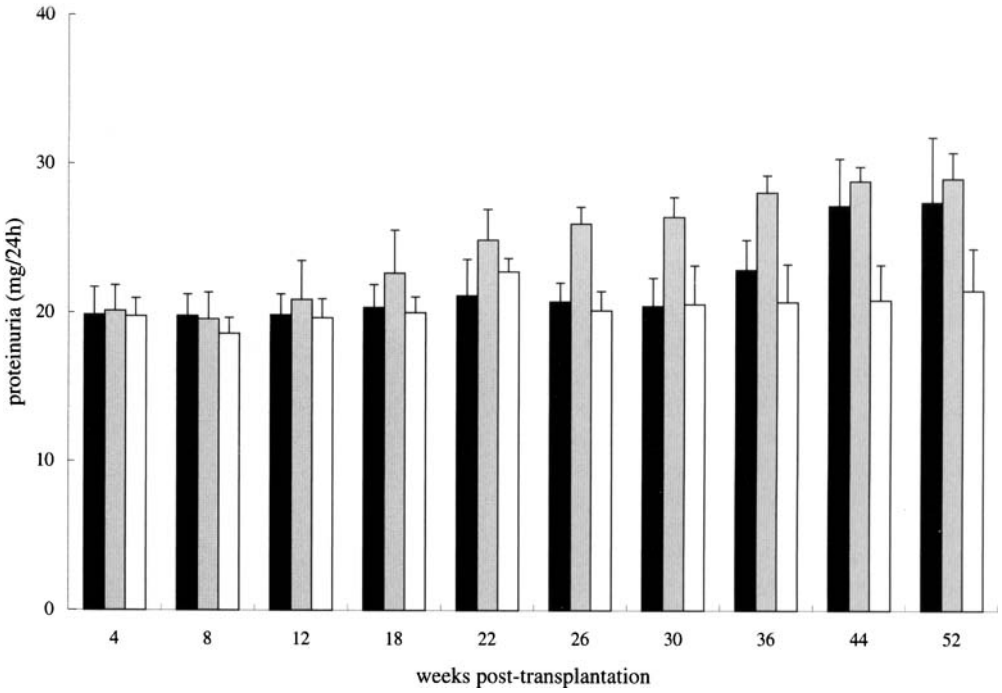

Fig. 1 Proteinuria in renal isografts (■), unilaterally nephrectomized (▒), and native controls (□). Isografts had a stable proteinuria during follow up, and the excretion was similar to that of UNx rats

each incubation, slides were washed in PBS-Tween 20, 0.1%. A second layer, rabbit anti-mouse IgG (Dako) was then applied for 30 min and after washing, slides were incubated with the third layer, mouse peroxidase-anti peroxidase (Dako) for 30 min. After washing in PBS, the reaction was developed by the addition of Diaminobenzidine substrate (Dako) and the slides were counterstained in Mayer's hematoxylin for 40 s, washed, dehydrated, and mounted. The analysis was carried out blindly per experimental group. Positive cells were counted at 400x magnification using a calibrated micro-ocular grid in > 16 fields of view and expressed as number of positive cells /0.1 mm^2. MHC class II and ICAM-1 expression was quantified on a 0-to-3 scale (0 = none, 1 = mild, 2 = moderate, 3 = dense).

Statistical analysis

Statistical analysis was performed using the Kruskal-Wallis one way ANOVA followed by the Mann-Whitney test, and $P < 0.05$ was accepted to be significant. The results are expressed as mean ± SD in the text and tables, and as mean ± SEM in the figures.

Results

Renal function

Isograft recipients had a minor, constant excretion of urinary protein during the 52 weeks follow-up, which did not differ from that in UNx or native rats during the whole observation period (Fig. 1).

The GFR (per 100 g BW) in native control rats remained constant during follow-up. Unilateral nephrectomy led to a significant reduction of the GFR, which was about 80% of that in native rats at the first measurement, 4 weeks post-transplantation. Transplantation of one kidney did not lead to a further reduction: the GFR of isografts did not differ from UNx rats at week 4 post-transplantation. After the initial reduction of GFR in UNx and isografts, it did not further change during the next 48 weeks follow-up (Fig. 2).

The diastolic and systolic BP measured at week 52 did not differ between the isografts, UNx-group, and native controls (Table 1).

Kidney weight/body weight ratio

A 50% reduction of the nephron number by nephrectomy led to compensatory hypertrophy of the remaining kidney: At week 52, the relative kidney weight of the UNx-group was similar to that of native controls (0.53 ± 0.06 vs 0.53 ± 0.03 g kidney/100 g BW). Kidney transplantation led to the same degree of compensatory hypertrophy: Isografts had a relative kidney weight that

Table 1 Systemic blood pressure in native, unilaterally nephrectomized rats and in rats with a syngeneic kidney transplant, measured intra-arterially at week 52 after transplantation

Group	*n*	Diastolic BP	Systolic BP (mm Hg)
BN	5	87 ± 5	117 ± 6
BN-UNx	8	86 ± 4	117 ± 3
BN-isografts	5	80 ± 4	115 ± 3

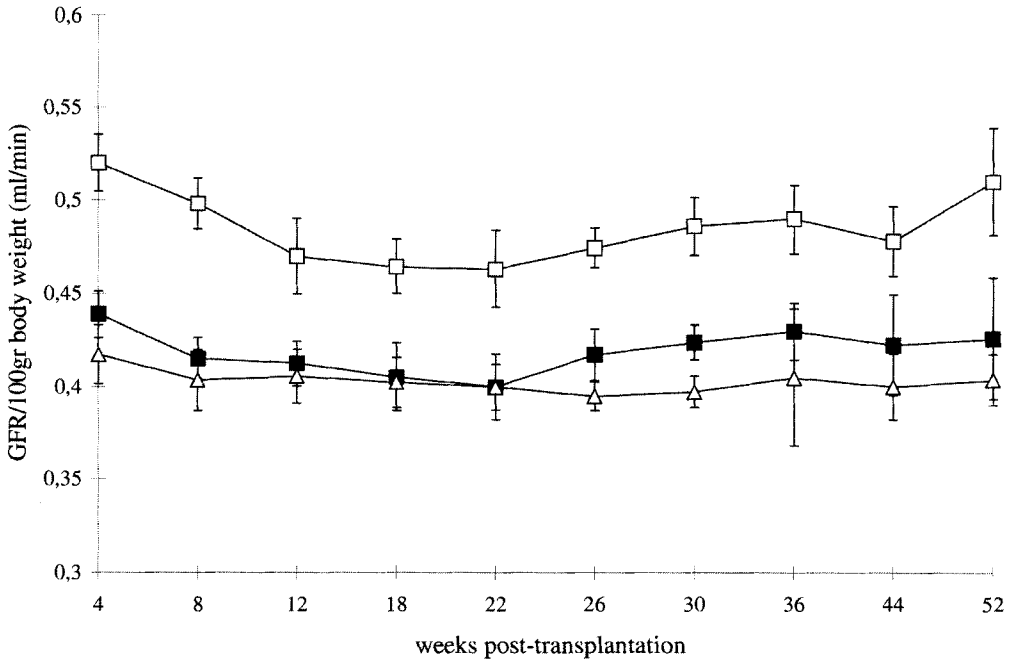

Fig. 2 Relative glomerular filtration rate in renal isografts (■), unilaterally nephrectomized (UNx) △) and native controls (□). Isografts and UNx had similar GFRs, which were about 80% of that in native controls. * = $P < 0.05$ vs UNx at the same time point; *Tx* transplantation

did not differ from that of UNx-rats (0.48 ± 0.04 vs 0.53 ± 0.06 g kidney/100 g BW).

Morphology

Renal isografts had a few focal mononuclear cell infiltrates that were also present in UNx and native kidneys. At week 52, the isografts demonstrated very mild glomerular changes. About 8% of the glomeruli were sclerotic, whereas mesangial matrix expansion with basement membrane thickening was evident in about 25% of the nonsclerotic glomeruli (BANFF: 1.4 ± 0.5). In all isografts, some areas of tubular atrophy were seen (BANFF: 1.0 ± 0). Interstitial fibrosis and intimal hyperplasia was not observed. The total BANFF score for isografts was 2.4 ± 0.5. UNx-kidneys demonstrated an identical morphology: 7% of the glomeruli were sclerotic and a quarter of the nonsclerotic glomeruli showed glomerulopathy (BANFF: 1.3 ± 0.5). As in the isografts, some tubules were atrophied, leading to a total BANFF score of 2.2 ± 0.4. Native control kidneys showed significantly less glomerulosclerosis (5%), but the percentage of nonsclerotic glomeruli having mesangial matrix increase did not significantly differ from UNx-rats. In some kidneys, minimal areas of tubular atrophy were present. Interstitial fibrosis and intimal hyperplasia were not seen in the controls. The total BANFF score was 1.5 ± 0.6.

Immunohistology

Native BN-kidneys showed some $CD4^+$ cells and $ED\text{-}1^+$ macrophages predominantly located in the interstitium (Table 2). MHC class II was expressed on the proximal tubules and by infiltrating cells. ICAM-1 expression was present on the glomeruli, the peritubular capillaries, and on some cells located in the interstitium. The numbers and patterns of infiltrating cells and the cell surface molecules in UNx-kidneys were comparable to those of native kidneys. On the contrary, the numbers of both $CD4^+$ cells and macrophages in kidney isografts were significantly higher, which was predominantly (70%) due to infiltration in the glomeruli. Concurrently, MHC class II expression was seen, and ICAM-1 expression was upregulated in the glomeruli. In the interstitium, MHC class II expression was upregulated.

Discussion

Both the etiology and pathophysiology of CTD are poorly understood. Recently, attention has been directed to non-allogeneic stimuli causing similar functional and morphological changes as seen in allogeneic ones. To further understand the importance of these alloantigen-independent factors, we investigated the impact of the transplantation procedure *per se* on the development of CTD in a single, syngeneic kidney transplant.

The main finding of the present study is that transplantation of a kidney in itself does not lead to late functional and morphological changes. At week 52, pro-

Table 2 Cellular infiltrates, MHC II and ICAM-1 expression in renal isografts, unilaterally nephrectomized (UNx) and native controls. Cells counts are expressed as mean ± cells/0.1 mm^2; MHC II and ICAM-1 expression were quantified on a 0–4 scale. * = $P < 0.05$ vs. UNx and native controls

	Isograft	UNx	Native control
$CD45^+$ leucocytes	24 ± 3 *	14 ± 1	14 ± 3
$CD4^+$ T cells	20 ± 3 *	13 ± 1	11 ± 2
$CD8^+$ T cells	4 ± 1	3 ± 1	4 ± 1
$ED1^+$ monocytes/macrophages	12 ± 3 *	6 ± 1	5 ± 1
MHC class II: proximal tubules	2.0	1.0	1.0
peritubular	1.5	1.0	1.0
glomeruli	0.5	0	0
ICAM-1: peritubular	1.5	1.0	1.0
glomeruli	2.0 *	1.0	1.0

teinuria, GFR, and the systemic BP were similar in isografts and UNx rats. Renal morphology was also identical: minor glomerulosclerosis, some glomerulopathy and tubular atrophy, without interstitial fibrosis and intimal thickening. These findings conflict with those of Tilney and co-workers, who reported that after 32 weeks Lewis renal isografts developed functional and morphological changes, including marked intimal hyperplasia, thereby resembling CTD [13]. These changes appeared to be induced mainly due to ischemia. Clamping the renal vessels of a unilaterally nephrectomized Lewis rat caused functional deterioration at 1 year identical to the isografts, whereas a nephrectomy in itself resulted in minimal late changes. In concordance with our data, other investigators using DA or AS rat strains [16, 5], did not observe intimal thickening after syngeneic kidney transplantation either, although the observation period was not as long as ours. Since there appear to be no differences in surgical procedures, it is conceivable that both ischemic injury and the subsequent response are strain dependent. This phenomenon which has been demonstrated by Ibrahim *et al*: 60 min ischemia in kidneys of different rat strains led to different numbers of infiltrating T-cells and macrophages, and different expression of MHC class II on tubular epithelial cells [4].

This study also demonstrates that a 50% reduction in nephron number by nephrectomy did not lead to progressive deterioration of function and morphology of the remaining kidney. Since glomerular filtration was 80% of that in native BN-rats, and thus hyperfiltration occurred, hyperfiltration did not lead to late renal injury. In addition, the normal expression of ICAM-1 and MHC class II, and the virtual absence of infiltrating cells in the glomeruli further indicate that a BN kidney is resistant to hyperfiltration-induced injury. Since UNx Lewis rats showed increased protein excretion, increased ICAM-1 and MHC class II expression at week 52 [13], this could reflect different susceptibilities to hyperfiltration. Kingma *et al* demonstrated different glomerular hemodynamics in response to subtotal renal ablation in Lewis and Fisher rats [6]. Also, the age at time of nephrectomy and the dietary protein intake have been demonstrated to be important determinants for the life span of a single kidney [1].

In conclusion, transplantation of a kidney subjected to merely 30 min ischemia does not lead to late renal injury. The BN-kidney may be important in further unraveling the pathophysiology of CTD: Its relative resistance to perioperative ischemic injury enables to disthinguish clearly between alloantigen-driven processes and the contribution of non-alloantigen-specific factors to the development of CTD.

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