Chronic transplant dysfunction

Etiological and pathophysiological aspects

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Proefschrift

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Aan mijn ouders
Aan Geeske en Job

Preface

Organ transplantation has saved the life of many people throughout the world, who suffered from end-stage organ failure. The University Hospital Rotterdam-Dijkzigt, is one of the Dutch organ transplant centers, in which kidney, heart and liver transplantation are performed. In close conjunction with hurdles encountered in clinical organ transplantation, experimental transplantation research has been for a long time one of the points of interest of the Department of Surgery. Experimental work in small and large animal models to overcome the thresholds to successful intestinal transplantation has been of special interest for years. My scientific curiosity for the transplantation-field was provoked during participation as a student in a research project at the Laboratory for Experimental Surgery to investigate xenogeneic islet transplantation as therapy for diabetes mellitus.

In addition to search for new transplant modalities, we also try to improve long-term graft survival as data show that clinical organ transplantation has not yet achieved its full potential on the long-term. Beyond one year post-transplantation, the half-life of organ grafts has little changed since the beginning of the transplantation-era. Long-term graft survival is predominantly hampered by chronic transplant dysfunction (CTD). So far, no drugs can prevent CTD and a therapeutic strategy is not within hand's reach, since causes and mechanisms leading to CTD are poorly known. Retransplantation is at present the only effective therapy.

In 1995, I first started experiments at the Laboratory for Experimental Surgery to gain insight into the etiology of CTD: The role of ischemia and surgery was studied in an aorta transplantation model. Participating in ongoing experiments, at the Departments of Medicine and Surgery, Monash University, Melbourne, Australia I investigated the pathogenesis of CTD after small bowel transplantation in a rat model. From a clinical point of view, I continued experiments on the pathogenesis in the kidney and studied the effect of ischemia, surgery and genetics. These studies were carried out in close collaboration with the Departments of Paediatric Surgery and Pathology, Erasmus University and with the Department of Nephrology, University Hospital, Essen, Germany.

This thesis outlines the problem of CTD, presents the results of the experimental studies mentioned above, and finally discusses the results.

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General introduction
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1.1 The problem of chronic transplant dysfunction

Since it was first shown in 1954 that successful transplantation of a healthy kidney could completely rehabilitate an individual with renal failure, transplantation of several organs has become an increasingly successful medical treatment for those with end-stage organ failure. In 1998, in only the Eurotransplant area, more than 3000 kidneys, 750 hearts, about 1000 livers, 230 lungs and about 100 pancreas from cadaver donors were transplanted.[1] Worldwide, 56 intestinal transplantations were performed in 1996.[2] The short-term results after clinical organ transplantation have improved progressively, principally due to refinements in tissue typing, advancements in organ preservation, operative techniques and ancillary care, more effective immunosuppressive agents, and better monitoring after engraftment. For example, one year survival of cadaveric kidneys has increased from approximately 50% by the end of the 1960s to about 85% nowadays,[3] and for living-related kidneys from 80% to 90-95%.[4]

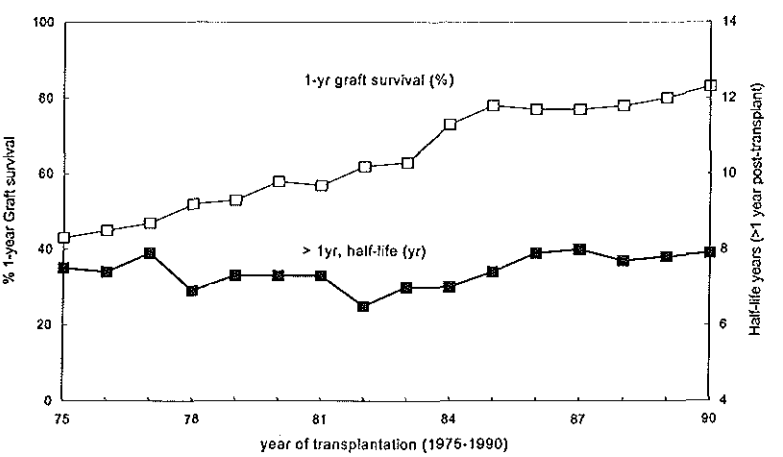

Figure 1. Derived from: Gjertson DW. Survival trends in long-term first cadaver-donor kidney transplants. in: Clin Transplant 1991. Terasaki P (ed), UCLA Tissue Typing Laboratory, Los Angeles, CA: pp225-235)

Despite the strongly improved early results, clinical transplantation has not achieved its potential as a long-term treatment for disease. Beyond one year the annual rate of graft loss has not improved as much as one year results. The half-

life of cadaver kidney allografts, for instance, has remained consistently at 7.5-9.5 years (Figure 1),[5,6] although the most recent data indicate that there is a slight improvement in half-life after the first year.[7] Other organ transplants generally show comparable results, with exception of the liver, which shows more favourable long-term results.[8,9]

Although a series of factors including recurrent and *de novo* disease, drug toxicity, acute rejection and non-compliance may contribute to late graft loss, chronic transplant dysfunction (CTD) is the most important, single cause of late graft deterioration and failure. Kidney graft loss is in 35 to 58% due to CTD,[10,11] more than 70% of lung allografts had CTD 5 years post-transplantation,[12] more than 50% of the heart transplants had severe coronary arteriosclerosis at five years,[13,14] and about 9-26 % of graft loss of liver transplants was due to CTD.[15-17]

There is still no treatment to inhibit or prevent CTD, and a logic therapeutic strategy is not within hand's reach, since its etiology and pathophysiology are poorly known. Until now, re-entry to dialysis in case of a kidney graft or direct retransplantation are the only effective therapies, the latter thereby heavily contributing to the problem of donor shortage.

1.2 Definition of chronic transplant dysfunction: functional and histological characteristics

CTD is the phenomenon in solid organ transplants in which a gradual deterioration of graft function occurs months to years after transplantation, eventually leading to graft failure, and which is accompanied by characteristic histological features.

Clinically, CTD in kidney grafts manifests itself as a slowly progressive decline in glomerular filtration rate, usually in conjunction with proteinuria and arterial hypertension.[18,19] In heart transplants, CTD presents itself with congestive heart failure, acute infarction, arrythmias and most dramatically, as sudden death.[20] The diagnosis of liver CTD should be based upon clinical evidence of chronic liver disease consisting of persistent enzyme abnormalities, elevated bilirubin, diminished synthesis of protein and blood clotting factors.[21] Intractable diarrhoea and weight loss are the accompanying symptoms in intestinal transplants with CTD.[22]

The cardinal histomorphological feature of CTD in all parenchymal allografts

is fibroproliferative endarteritis.[23] Other terms frequently used for this lesion are transplant arteriosclerosis, graft vasculopathy, graft vessel disease or intimal hyperplasia. The vascular lesion affects the whole length of the arteries, although in a patchy pattern. There is concentric myointimal proliferation resulting in fibrous thickening and the characteristic 'onion skin' appearance of the intima in small arteries.[23] Other findings include endothelial swelling, foam cell accumulation, disruption of the internal elastic lamina, hyalinosis and medial thickening, and presence of subendothelial T-lymphocytes and macrophages.[24] In addition, a persistent focal perivascular inflammation is often seen.

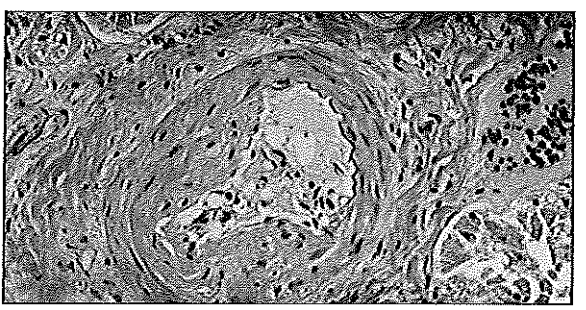

Figure 2. Intimal proliferation in small artery of a kidney transplant

Although intimal hyperplasia is very specific for CTD, the diagnosis of CTD in biopsies of allografts is frequently based on other, less specific abnormalities, since intimal hyperplasia is very patchy and affects mainly arteries larger than those seen in biopsies.

In the cardiac allograft, concentric intimal hyperplasia is the only histological feature of CTD. The myocardium appears to be no primary target in the CTD process: The small, triangular-shaped fibrosis in the heart muscle is the remainder of an infarction as result of vessel occlusion.[25]

In addition to vascular changes, a CTD kidney shows parenchymal manifestations, including interstitial fibrosis, tubular atrophy and glomerulopathy. Chronic transplant glomerulopathy - duplication of the capillary walls and mesangial matrix increase - has been identified as a highly specific feature of kidneys with CTD.[26] Less specific lesions are glomerular ischemic collapse, tubular atrophy and interstitial fibrosis. Furthermore, peritubular capillary

basement splitting and laminations are associated with late decline of graft function.[27] The criteria for histologic diagnosis of CTD in kidney allografts are internationally standardised in the BANFF scheme for Renal Allograft Pathology.[28]

CTD in the liver is characterised by intimal hyperplasia in medium-sized vessels, similar to that seen in other organs. In biopsy samples, diagnostic indicators are non-specific hepatocellular changes, centrilobular ballooning, degeneration, atrophy, fibrosis and canalicular cholestasis, especially seen in the centrilobular zone.[21,29-31] Sometimes overlapping with the vascular changes is the loss of bile ducts, called 'chronic vanishing bile duct syndrome' or 'ductopenic rejection'.[21,30,32]

The histopathological hallmark of CTD in the lung is bronchiolitis obliterans,[33] a lesion characterised by submucosal fibrosis and chronic inflammation ultimately leading to obliteration of the terminal airway. Obliterative endarteritis, if found, is diagnostic of CTD of the pancreas. Acinar tissue loss, fibrosis and absence of pancreatic islets are frequently found.[34] In human small bowel allografts, CTD is evidenced by patchy intimal hyperplasia and parenchymal lesions such as distorted mucosal architecture with focal necrosis and ulceration, villous blunting, loss of goblet cells, and focal lamina propria fibrosis.[35,36]

Kidney	Heart	Intestine
Low-grade inflammation	Low-grade inflammation	Inflammation
Arteriosclerosis	Arteriosclerosis	Arteriosclerosis
Glomerular basal membrane thickening and sclerosis	Fibrosis	Mucosal atrophy
		Muscular hyperplasia
Tubular atrophy		Fibrosis
Fibrosis		

Table 1. Histopathology of chronic transplant dysfunction in different organs

Until now, such typically functional and histologic changes of allografts are often diagnosed as 'chronic rejection'. However, the designation 'rejection' presumes a host alloimmune responsiveness to be basis for these changes. Since

there are indications that non-alloimmune mediated factors involved in organ transplantation can cause similar functional and histopathological changes, calling the process on the whole chronic rejection is not satisfactory, both from a mechanistic and therapeutic point of view. When seen as a pure immunologic process, immunosuppressive drugs are the goal to treatment, whereas non-alloimmune mediated processes need other treatment. As long as the result - dysfunction and characteristic histologic changes - cannot be exclusively attributed to an alloimmune-mediated pathway, it is recommended to call the process CTD that leaves any causative factor out of consideration.

1.3 Etiology of chronic transplant dysfunction

In 1963, Porter *et al.* reported four human cadaveric kidney allotransplants in which striking obliterative vascular lesions developed a few months after transplantation.[17] All patients had experienced early episodes of acute rejection, and the subsequent vascular lesions were thought to have an immunological basis. These cases suggested that the process of allograft rejection can evolve from early acute cellular infiltration of the engrafted organ to a more chronic process, ultimately resulting in intimal arterial thickening, with interstitial fibrosis. However, until now the cause of CTD remains ill defined. Two working hypotheses are proposed to understand the process: 1) the phenomenon leading to CTD is the result of an ongoing host alloimmune response. 2) Non-alloimmune responses-to-injury, such as ischemia, can cause or aggravate the process.

1.3.1 Alloantigen-specific factors

Several clinical and experimental data indicate that CTD is the result of the recipient 's immune response to incompatible donor tissue antigens. In this view, the relationships between the following identified risk factors and CTD all reflect an alloimmunologic mechanism: 1) Histoincompatibility, 2) Acute rejection, 3) Suboptimal immunosuppression/non-compliance, and 4) Anti-donor specific antibodies

Histoincompatibility

Antigenic disparity in humans between donor and host is associated with the occurrence of CTD, as demonstrated in kidney, heart, and lung transplant studies. In these multicenter studies, long-term graft survival appeared to be strongly correlated with the degree of histocompatibility between donor and recipient.[3,6,38-40] Cadaveric kidneys with zero HLA-mismatches have a half-life of 13.2 years compared to 7.0 years in grafts with six-allelic mismatches.[6] Interestingly, some large unicentre studies are unable to demonstrate the benefit of histocompatibility matching for the development of CTD in kidneys and hearts, independently of the effect of acute rejection.[41,42] It is presently unclear whether matching directly affects the development of CTD or whether this results from a decreased incidence of acute rejection episodes.[40,43-45]

Acute rejection

Graft survival studies from both uni- and multicentres show a strong correlation between acute rejection episodes and the lifespan of a graft.[46-49] For instance, Matas *et al.* showed in a group of 278 cadaver kidney graft recipients that a single rejection episode in the first post-transplant year reduces the estimated graft half-life from 33 years to 22 years, whereas multiple rejections or a single rejection after the first year decreases the half-life to less than 5 years.[48] More specific, several retrospective analyses of organ grafts with CTD demonstrate that acute rejection is strongly related to the development of CTD in all types of organ transplants.[35,41,42,50-54] Basadonna *et al.* reported that in a cohort of 205 cadaver renal transplant recipients, the incidence of biopsy-proven CTD was 0% in the 109 patients without acute rejection, 36% in the 69 patients with an acute rejection within the first 2 months after transplantation ($p<0.001$), and 63% in the 27 patients with acute rejection 60 days after transplantation ($p<0.001$).[55] Other clinical studies have corroborated and refined these findings: The onset, frequency, and severity of an acute rejection episode are independent risk factors for CTD.[42,47,50,55,56] Acute rejections with complete functional recovery do not have a deleterious effect on the long-term outcome,[57,58] whereas an increased baseline serum creatinine after treatment of an acute rejection episode is associated with CTD.[49,59] In addition, the vascular type of acute rejection appears to be a stronger risk factor for the occurrence of CTD than interstitial rejection.[60] Thus from clinical studies, the impact of a single acute rejection episode with a complete functional recovery in the early posttransplant period is minimal, whereas late

and more severe acute rejection episodes are more likely to develop CTD.

Experimental studies in kidney, heart and lung transplantation models confirm these clinical observations.[61-64] For instance, Yilmaz and Häyry showed in rat kidney allografts that intimal hyperplasia increased from 0.5±0.4 in a scale from 0 to 3 in the no-rejection group to 1.7±0.9 in the group with one rejection ($p<0.01$), to 2.2±0.3 in rats with 2 rejections ($p=0.0001$), and to 2.2±0.5 for rats with 3 or 4 rejections ($p<0.01$).[61] Nonetheless, acute rejection is not a prequisite for CTD: patients also develop CTD without prior acute rejection episodes.[50,55,65] Reviewing the literature it can be stated that at present acute rejection is the most consistently identified clinical risk factor for the occurrence of CTD.

Suboptimal immunosuppression and noncompliance

A low dose of maintenance Cyclosporin (CsA) medication in some clinical studies has been associated with CTD,[16,66,67] but not in others.[68] At 5 years post-transplantation, the percentage of recipients who were free of CTD as demonstrated by biopsy was 86% for those using CsA > 5mg/kg/day versus 77% for those on <5 mg/kg/day.[67] Additional evidence that CTD may be related to inadequate immunosuppression was provided by the histopathological studies of Isoniemi *et al.*[69] They found that CTD-lesions were less apparent in patients given protocols of triple-versus double-dose immunosuppressive therapy.

Experimentally, previous investigations at our own institution demonstrated that in a rat aortic allograft model both high dose CsA as well as Rapamycin were able to abolish the inflammatory response, and concomitantly inhibit the generation of intimal lesions during the 4-weeks follow-up period.[70] Other groups also reported that high dose of CsA alone[71] or in combination with leflunomide[72] or with methylprednisolone and azathioprine[73] can prevent CTD in allografts. However, in man it would be impossible to maintain high doses of immunosuppressants on the long-term, because of toxic side effects of the various drugs, e.g. nephrotoxicity caused by CsA and FK 506.[74]

Noncompliance also indicates that CTD may result from inadequate immunosuppression.[75-77] In a study by the Minneapolis group 34% patients were noncompliant and this was associated with late deterioration of graft function.[78]

Anti-donor antibodies

Many studies have shown that following renal,[79-83] cardiac,[84-87] or hepatic transplantation,[88] the majority of patients produce antibodies. Both preformed

antibodies reactive against donor tissue and antibodies after transplantation against HLA class I antigens and against tissue (endothelial cells, smooth muscle cells) are found. A correlation between antibody and CTD, however, is not consistently found. [66,82,83,88-92]

No difference in panel-reactive antibody levels was found between patients whose grafts were still functioning versus those who lost their graft due to CTD.[67,90] Likewise, Hosenpud *et al.* could not demonstrate differences between the presence of IgM antibodies against endothelial cells of kidney grafts with and without CTD.[91] Other investigators, however, observed significantly more anti-donor reactivity against both HLA class I and II in sera of patients with biopsy-proven CTD in kidneys (94.4%) than in sera of patients with a normal functioning graft (12.8%).[93] Likewise, in 70% of the liver allografts with CTD patients had non-HLA anti-smooth muscle and anti-nucleus antibodies, which were not present in patients with a healthy liver transplant.[88]

Experimentally, Paul and colleagues demonstrated IgG antibodies against the glomerular and tubular basement membrane, the mesangial cell, and endothelial cell antigens in sera of rats with a kidney allograft with CTD, whereas such antibodies were not found in sera from animals that had received a syngeneic graft.[94,95] In other experimental models of CTD, the presence of antibodies was noted in areas with intimal hyperplasia.[96,97]

1.3.2. Non-Alloantigen associated factors

In the late 80-ies, attention has been redrawn to the fact that in the pre-immunosuppression era even human kidneys transplants between identical twins developed late morphologic changes. Two-third of these kidney isografts developed glomerular lesions - hypercellularity, increase of mesangial matrix and immunoglobulin deposition - between 2 months and 16 years post-transplantation that was classified as recurrence of the original disease, glomerulonephritis.[98] Two of these grafts with glomerular lesions developed in a later stage additional vascular lesions. It was also suggested that such changes observed in human renal isografts might have been a consequence of the transplantation injury *per se*.[99]

Nowadays, based upon clinical and experimental data from different organ transplants, surgical injury and other, non-alloimmune specific factors related to the donor and the graft have been associated to the development of CTD.[100-102]

These risk factors include: ischemia, brain death, viral infections, hyperlipidemia, hypertension, age, gender, race, and the amount of functional tissue.

Alloantigen specific factors	Non-alloantigen specific factors
Histoincompatibility	Ischemia
Acute rejection	Brain death
Suboptimal immunosuppression	Infection
Anti-donor antibodies	Hyperlipedemia
	Age
	Gender
	Race
	Size

Table 2. Risk factors for chronic transplant dysfunction

Ischemia

In clinical transplantation it is still unclear if ischemia participates in the development of CTD. Whereas some studies reported that prolonged cold ischemia reduces graft survival,[3,44,103] other centres found no correlation.[42,104] For instance, the UNOS registry showed that preservation for >24 hours significantly impaired late kidney graft survival rates compared to cold ischemic times between 0 – 24 hr.[3] In cardiac transplants, a prolonged ischemic time was a risk factor for transplant arteriosclerosis.[105]

Experimental transplant studies have been demonstrated that ischemia can cause CTD-like lesions in the absence of allogenicity. Tilney and co-workers demonstrated that rat kidney isografts develop the same functional and morphologic changes as allografts, including vasculopathy, albeit over a much longer time interval.[101] These changes appeared to be triggered mainly due to ischemia. Clamping the renal vessels of a unilaterally nephrectomized rat caused functional deterioration identical to isografts at 1 year post-surgery. Similarly, on the long-term syngeneic aorta and heart transplants develop intimal hyperplasia.[106,107] In isografts the degree of intimal hyperplasia correlates with the duration of the ischemia period.[106,107]

Nonetheless, it is much less clear whether the length of the ischemic period plays

a role in the onset of CTD in allografted organs. Whereas Hayry's group showed that in renal allografts a cold ischemic time of 60 minutes led to increased intimal proliferation and glomerulosclerosis compared to kidneys subjected to 30 min cold ischemia,[108] in heart and aortic allografts the duration of the cold ischemic period did not have an influence on the degree of CTD.[106,109,110]

It has also been suggested that in allografts the effect of ischemia on CTD is indirect: Ischemia might predispose for acute rejection, the latter being the most consistent clinical risk factor for CTD. Organ grafts with prolonged cold ischemia or with delayed graft function, which is a parameter that correlates with the ischemic period, experience more often an early acute rejection episode than grafts that functioned immediately.[103,111-114]

Brain death

The striking divergence in clinical long-term results between kidney grafts from cadavers and those from living-related and unrelated donors[115] has drawn attention to the healthiness of an organ before procurement. The hypothesis has been put forward that brain death activates surface molecules on peripheral organs via cytokines: In brain death donors, increased serum cytokine levels, such as IL-1β, IL-6, IL-8, TNF-α, and RANTES are found before organ procurement.[116] In experimental models of brain death, peripheral organs have increased endothelial cell activation[117,118] and a more accelerated tempo of acute rejection in organs from brain death animals is observed.[119,120] The relevance for CTD still has to be proved.

Viral infection

Whereas infection with cytomegalovirus (CMV), a member of the herpes virus family has shown to be related to CTD in cardiac, liver, and lung transplantation,[52,53,121,123] its association with CTD in kidney transplants is not yet clear. A multivariate analysis on risk factors for CTD performed on 675 renal allograft recipients at Hennepin County Medical Centre in Minneapolis showed no difference in the incidence of CMV infection in patients who did or did not loose their grafts to CTD.[41]

CMV infection has been experimentally identified as promotor of CTD in aorta, kidney and heart transplants.[124-126] CMV infection directly affects intercellular adhesion molecule-1 (ICAM-1) expression on endothelial cells[125] and induction of MHC class II antigens is observed, together with a prolonged and

increased acute cellular infiltration of T cells and macrophages.[126,127] In both hearts and kidneys, a more pronounced intimal hyperplasia was seen.[125,127] Similarly, administration of lipopolysaccharide, an endotoxin of *Escherichia coli* to a rat model of kidney CTD led to an earlier expression of ICAM-1 which correlated with an accelerated pace of CTD.[128,129]

Hyperlipidemia

In patients bearing kidney or heart transplants, hyperlipidemia is considered as a risk factor for allograft arteriosclerosis.[130-132] However, different opinions exist.[133,134] The relevance of hyperlipidemia in animal transplant models has also been a matter of controversy. Alonso *et al*,[135] Fellstrom *et al*,[136] and Tanaka *et al*[137] have reported enhancement of arteriosclerosis by hyperlipidemia, whereas Mennander *et al*,[138] and Adams *et al* [139] could not confirm these observations.

Hypertension

Systemic hypertension in clinical kidney and heart transplants is associated with CTD.[140-143] In heart transplant recipients, hypertension was associated with an earlier onset of CTD.[143] In renal allograft recipients, hypertension is a common event (75%), although its role as a causative factor or a consequence of renal dysfunction is difficult to define since a vicious circle is created where the worsening of one parameter leads to worsening of the other.[144]

Experimental studies showed that systemic hypertension accelerates CTD in kidney allografts,[145] whereas antihypertensive drugs inhibited the progression of chronic allograft dysfunction.[146] Similarly, in rat aortic transplants, hypertension was associated with a significant increase of intimal thickness, while ACE-inhibitor was able to decrease systolic blood pressure by 30%, and concomitantly reduce intimal lesions by 40%.[147]

Donor age

Donor age is a controversial risk factor. Some investigators have found that grafts from donors over 60 years of age are associated with poorer survival rates.[148-150] Cardiac transplants from an older aged donor had an earlier onset of CTD.[102,151]

Gender

In male recipients, solid organ grafts appeared to be more vulnerable for the development of CTD. In cardiac allografts the onset of arteriosclerosis was earlier

in males than in females[102] and another study reported that the prevalence of CTD was higher in male than in female recipients: 30% versus 50% free of coronary artery disease at 5 years ($p = 0.01$).[152] The UNOS Transplant Registry reported a similar observation for kidney grafts.[3]

Experimental studies have corroborated the observations: In rat syngeneic aorta transplants, the female gender protects against myointimal hyperplasia.[153] This gender effect could reflect oestrogen. Oestrogen protects against cardiovascular disease and, it has been demonstrated that oestradiol effectively inhibits transplant arteriosclerosis in experimental models.[154-156]

Race

Long-term survival of cadaveric renal transplants appeared to be related to race: Five-year graft survival rates were 66% for Asians, 61% for Caucasians and Hispanics, and 47% for Black recipients.[157] Black recipeints of heart transplants developed earlier CTD than non-blacks.[102]

Functional tissue mass

One risk factor reserved to the kidney is the contribution of reduced numbers of nephrons to the progression of CTD. In non-transplant models in the rat, it is well established that kidneys with significantly reduced numbers of nephrons, such as in the 'remnant kidney model', develop glomerulosclerosis, tubular atrophy and interstitial fibrosis in response to an increased workload of the remaining nephrons, i.e. hyperfiltration.[158,159] In a chronic kidney allograft model, Heemann *et al.* demonstrated that reduction of renal mass led to earlier onset of CTD and shortened survival. Moreover, isografts and non-transplanted, ablated kidneys having only 1/6 of total mass experienced proteinuria in a same tempo as allografts, whereas 2/6 or 3/6 nephrectomized native and isografted kidneys had negligible damage.[160] Thus, the reduction of functioning renal mass accelerated the changes characteristic for CTD, and after a substancial reduction, hyperfiltration plays an overriding role in further deterioration. In the same animal model, the inadequate nephron supply was further investigated by comparing one allograft plus one retained native kidney, or two allografts with one allograft with bilateral nephrectomy. Recipients with 2 kidneys had less allograft injury and had no hyperfiltration and normal glomerular pressures whereas solitary grafts revealed hyperfiltration and increased glomerular pressures.[161]
Nonetheless, in clinical kidney transplantantion the significance of a mismatch

between donor nephron supply and functional metabolic demand of the recipient in the development of CTD is unclear. Poorer survival of grafts from very young, elderly, black or female donors compared to grafts from donors aged 15-55, non-black or male donors has been ascribed to hyperfiltration damage.[162] None of these CTD-prone conditions, however, are uniformly found. Miles *et al.* did not find that the donor kidney size was different in patients who lost their graft due to CTD compared with those with stable function.[163] Others also did not see differences in CTD between pediatric kidney recipients and adult-kidney recipients.[164] Paired pediatric kidney transplantation did not improve renal function compared to small single pediatric kidneys.[165]

1.4 Pathophysiology of chronic transplant dysfunction

As outlined in paragraph 1.2, CTD is characterized by morphological evidence of destruction of the transplanted organ.[21] The common denominator in all parenchymal organs is the development of intimal hyperplasia. Whether the parenchymal changes with fibrosis occurs secondary to gradual arterial insufficiency and ischemia or develop from ungoing subclinical host immunologic attack or other factors remains undefined.
Immunohistochemistry of allografts with CTD has shown that T cells and macrophages are the predominant graft-invading cell types, with an excess of $CD4^+$ over $CD8^+$ T cells.[21,166-171] Increased expression of adhesion molecules (ICAM-1, VCAM-1),[172-174] and MHC antigens[173-174] are seen in allografts with CTD. Also, complement and immunoglobulin deposits is seen in areas with intimal hyperplasia.[96,172,174] Little consistent information is available regarding the expression of growth-regulating factors and their receptors in organ transplants with CTD. An increased TGF-β expression, however, is frequently found.[175-179]

The histologic lesions, including intimal hyperplasia, the infiltrating cells, upregulated adhesion molecules, and cytokines in organ transplants with CTD do not necessarily reflect an alloimmune-mediated response. As already mentioned, syngeneic transplants, ischemia- or mechanical-injured organs also show cell infiltration, upregulation of cytokines and develop CTD-like lesions.[101,107] Notwithstanding, the development of the lesions occurs much more rapidly in allografts, suggesting that alloimmune responses play a role.[101] The most consistent clinical risk factor 'acute rejection' also indirectly indicates that

an alloimmune response is involved in CTD and suggests that CTD is, for the main part, the result of insufficient immunosuppression. More evidence to support this hypothesis comes from experiments which have demonstrated that pretransplant immunizations with donor splenocytes accelerate CTD,[180] whereas manipulations aimed at induction of tolerance delay the process.[181,182]

In the following subparagraphs, we postulate the route through which an organ transplant may develop CTD.

1.4.1 Initial response-to-injury

Endothelial cell activation

The specific adhesion of cells to other cells or to particular tissues is a basic function of cell migration and recognition. Under normal conditions, contact between leukocytes and vascular endothelium is random if both cell types are inactive and at rest, the cells touch vessel walls indiscriminately.

In organ transplantation, the endothelial cells are activated by ischemia, surgical manipulation, and reperfusion injury, events inherent to the procedure. After ischemia and reperfusion, endothelial cells produce oxygen free radicals predominantly via the xanthine-oxidase pathway, which in vitro activate and damage the cells.[183] Upon activation, the endothelial cells retract and release increased amounts of the cytokines IL-1, IL-6, IFN-γ, TNF-α, the chemokines IL-8, macrophage chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 and MIP-1, colony stimulating factors, and multiple growth factors such as, platelet derived growth factors (PDGF), insulin growth factor-1 (IGF-1), transforming growth factor (TGF)-β, and pro-thrombotic molecules (tissue factor, plasminogen activator inhibitor). This secretion enhances migration of neutrophils, monocytes/macrophages and T lymphocytes to the site of injury.[185] The release of cytokines also led to upregulation of adhesion molecules on the vascular endothelium.[185,186] The proinflammatory cytokines IL-1 or TNF-α induce the expression of the adhesion molecules P- and E-selectin on the endothelium,[187,188] by which circulating leucocytes begin to adhere via binding to their surface carbohydrates.[189-191] Leucocytes are then triggered by the chemokines released by the endothelium, which causes upregulation of the affinity of the β2-integrin receptors LFA-1 and MAC-1 on their surface. This enables a permanent adherence of leucocytes to the endothelial adhesion molecules ICAM-1 and VCAM-1,[192,193] which expression is induced by the released cytokines IL-1β, IFN-γ, TNF-α.[194,195] Activated complement also plays a role in the

adhesion of neutrophils and monocytes to endothelium.[196] Finally, extravasation of leucocytes occurs to the extracellular matrix and graft tissue, presumably facilitated by activated complement[197,198] and oxygen free radicals, which increase the permeability between endothelial cells.[184]

The first cells that infiltrate the graft are neutrophils. They further aggravate the inflammatory response through release of oxygen free radicals and inflammatory mediators, including platelet activating-like factors and leukotrienes.

Direct evidence that oxygen free radicals, adhesion molecules, and neutrophils play a role in the pathogenesis of CTD is shown by interference studies.[199-202] One recent study, for instance, revealed that carotid allografts from donor mice deficient in ICAM-1 had a 52% reduction of intimal hyperplasia compared to controls.[203]

In addition to the increased expression of adhesion molecules on the endothelium, after reperfusion of a transplanted organ a dramatical upregulaton of MHC class I and II antigens on the endothelium occurs,[204-206] which appears to be induced by release of cytokines IFN-γ, TNF-α and TNF-β.[207] Alterations in tissue density of MHC class II antigens are likely to influence the alloimmune response against the tissue.[208] Parenchymal cells are also activated after ischemia. In non-transplanted kidneys MHC class I and II antigens are upregulated on the tubular epithelium.[205,209] Epithelial cells in lung autotransplants showed after cold ischemia a mild expression of MHC class II.[206]

$CD4^+$ T-lymphocytes infiltrate ischemic allografts, isografts and non-transplanted organs.[101,205,210] In addition, T cell associated cytokines, such as IFN-γ and TNF-α are produced[205,211] and blockade of the C28-B7 costimulatory pathway decreased early influx of T cells and expression of T cell associated cytokines.[212] The role for $CD4^+$ T-lymphocytes in ischemia has been elegantly demonstrated. In a liver ischemia model, $CD4^+$ T cell deficient mice had significantly less hepatic damage.[213]

This response to ischemic injury is initially independent from allogenicity: Heemann *et al.* demonstrated that the pattern of cellular infiltration and cytokine expression in both syngeneic and allogeneic cardiac grafts was similarly if not identically within the first 48-72 hr after engraftment.[214]
Thus as result of the transplant procedure, a complete network of cytokines is already activated, even before allogeneic reactions develop. Some pre-transplant conditions of both donor and recipient, discussed in paragraph 1.3 appear to aggravate this initial injury.

Alloimmune response

In transplant recipients, the recognition of histoincompatible MHC alloantigens will provoke an alloimmune response. Class I antigens, constitutively expressed on nucleated cells, interact with $CD8^+$ cells, and class II antigens, constitutively expressed on lymphoid cells and inducible on endothelial cells, macrophages and fibroblasts are recognised by $CD4^+$ cells.[215] Intact foreign MHC molecules on donor cells may be directly recognised by T cells, either in combination with an allopeptide or a selfpeptide,[215] which results in an exceptionally strong immune response. Frequencies of T cell precursors that respond to alloantigens are 10 to 100 fold higher than for other nominal antigens.[216] The common pathway of antigen presentation also takes place in allotransplantation: In draining lymph nodes and spleen, alloreactive T-cells recognise donor MHC as processed peptides presented by self-MHC molecules on recipient antigen presenting cells.[217] This is called 'indirect allorecognition'.

In allorecognition, the MHC antigen is bound to the T cell receptor. For activation of the T-cells, costimulatory pathways as the CD28 receptor on T cells with its ligand B7 and CD40 with its T-cell based ligand, CD40L are mandatory for the promotion of T-cell effector function and proliferation. The adhesion molecules ICAM-1, VCAM-1 and LFA-3 have also been shown to co-stimulate T cell activation. Once the $CD4^+$ T cell is activated, a cascade of events amplifies the alloimmune response: Secreted IL-2 leads to clonal proliferation of alloreactive cells and stimulates $CD8^+$ T cells to develop into mature cytotoxic effector cells. Release of cytokines such as IFN-γ, TNF-α, may further increase the expression of adhesion molecules, and MHC antigens on the endothelium, smooth muscle cells and parenchymal cells. IFN-γ is also responsible for the activation of macrophages, which together with $CD8^+$ cells are cytotoxic to the graft cells, leading to acute graft failure, when no immunosuppressive intervention is given to prevent or to overcome this $CD4^+$ T-cell mediated alloimmune response.

Despite inhibition of T cell activation by Cyclosporin, FK 506, or anti-IL-2 monoclonal antibodies, these therapies do not prevent the development of CTD in clinical transplantation, probably due to too low doses of these drugs. In experimental models, continuously high dosis of Cyclosporin A or blockade of CD28/B7 and CD40/CD40L costimulatory pathways decrease early infiltration and almost completely inhibit intimal hyperplasia in murine aortic and cardiac allografts.[69,70,218,219] The striking evidence that the $CD4^+$ T cell is involved in the genesis of intimal hyperplasia is elegantly exemplified by Shi *et al*: Carotid

allografts in mice which were genetical deficient for the $CD4^+$ T cell developed intimal thickening to only 40% of that seen in controls.[220]

Anti-donor specific antibodies

The cytokines IL-4, IL-6, and IL-10 released by activated $CD4^+$ cells are growth and differentiation factors for B cells. Activation of B cells may result in maturation into plasma cells with allospecific antibody production. Since immunoglobulin, complement, antigen-antibody complexes have been found in areas of intimal hyperplasia,[221-223] humoral activity has long been thought to be primarily responsible for CTD. A recent finding of upregulated immunoglobulin J chain in arteriosclerotic lesions suggests the presence of IgM- or IgA-producing plasma cells in such grafts.[224] Donor-specific antibodies are found against HLA antigens, endothelial cells, mesangial cells, glomerular and tubular basement membrane, smooth muscle cells and the nucleus.[80,181,225]

The precise significance of antibody deposition that mitigates over time, as shown in many animal models,[95,96,172] remains to be established. In experiments with SCID mice, which lack T and B cell mediated cellular responses passive transfer of anti-donor specific antibody was sufficient to produce graft arteriosclerosis with a perivascular mononuclear cell infiltrate in longstanding cardiac allografts.[226] Whereas some investigators found that the degree of intimal hyperplasia aortic and cardiac allografts in mice recipients with a defect of humoral antibody production was comparable to that seen in immunocompetent mice,[227] Russell *et al.* showed that cardiac allografts in B-cell deficient mice did not develop fibroproliferative arteritis.[228] These investigators also demonstrated that in two donor-recipient mice combinations in which anti-donor antibodies are generally undetectable, intimal fibrosis was uncommon, whilst these recipients became capable of producing fibrous lesions in allografted hearts when given anti-donor, class I antibody.[228] Similarly to Russell's report, Shi *et al.* showed that $CD4^+$ cells, humoral antibodies and macrophages together were necessary for intimal hyperplasia in a mice carotid allograft model. Arteries allografted into mice deficient in both T-cell receptors and humoral antibody showed almost no neointimal proliferation, whereas those grafted into mice deficient only in humoral antibody developed minimal intimal hyperplasia.[220]

The mechanism by which antibodies contribute to CTD is rather speculative. One recent study has shown that anti-HLA antibodies when sticked to their HLA class I antigen on cultured endothelial cells induce increased gene expression of

bFGF receptor and ligand binding, and a 4 to 6 fold cell proliferation, as it does for smooth muscle cells.[229] Marsh *et al.* hypothetized that IgG induces the accumulation, differentiation and subsequent cytokine production by intimal macrophages via crosslinking of FcR thereby preventing apoptosis of monocytes. FcR crosslinking induces the production of MCP-1 and IL-8, which can promote both macrophage and lymphocyt accumulation. [230,231]

1.4.2 Chronic response-to-injury

It is not clear why this response to the initial injury does not disappear over time, as seen in normal healing processes. In allografts, it is conceivable that the alloantigens are responsible for an ongoing cellular and/or humoral response. T cells decline to relatively low levels as the process enters its chronic phase, they and their products may continue to provide a persisting low grade immunologic response and ongoing subclinical injury to the graft's endothelium and parenchyma over time.[232] Since there is a continuous supply of donor allopeptides processed and presented by host professional antigen-presenting cells (dendritic cells, macrophages, B cells), self-MHC restricted T cells may perpetuate a chronic alloimmune response. Recently, Suciu-Foca and colloboratives demonstrated a persistent allopeptide reactivity in patients developing CTD.[233] The continued alloimmune recognition in long term graft recipients is evidenced by the presence of graft reactive cytotoxic T splenocytes in long term recipients of cardiac allografts.[234] Anti-donor specific antibodies may also persist a chronic alloimmunologic injury: Donor reactive alloantibodies in the recipient's circulation have been demonstrated long-term after engraftment.[82,220,235]

The significance of donor alloantigens on 'non-professional' antigen presenting cells, like the endothelial cells for T cell recognition is unclear. An indication that donor MHC class I and II antigens play a role in the chronic phase has been recently shown. Carotid allografts from donor mice deficient in MHC class II molecules showed a reduction of intimal hyperplasia formation of 33%, primarily due to a reduction in smooth muscle cell accumulation.[203]

In this hypothesis, the absence of such a continuous alloantigeneic stimulus in syngeneic transplants might explain the much more rapid development of CTD in allografts. Nonetheless, non-alloimmune specific factors are involved in the process. Ascessory factors mentioned in paragraph 1.3.2 can accelerate or inhibit the progress of the lesions.

Thus, the strenght of the initial trigger, the length of the trigger, and the presence

of additional factors, under which alloantigens, determine the onset and the pace of progress of irreversible chronic lesions

Macrophages

After activation, T cells produce amongst others the cytokine RANTES (Regulated upon activation, normal T cell expressed and secreted), a macrophage chemoattractant.[235] Macrophages invade the graft and become activated by IFN-γ. The continued presence, the activated state and the upregulation of macrophage associated cytokines in long-term allografts with CTD and in other chronic diseases with fibrotic features suggest a pivotal role for the macrophages.[168,169,178,237]

In organ transplantation, a demonstration of the importance of the macrophage was provided by the prevention of CTD by treatment with gammalactone, a synthetic inhibitor of macrophage activity, in a rat renal allograft model,[178] and by the observation that carotid allografts in mice deficient in macrophages, developed only small intimal hyperplasia.[220]

Activated macrophages produce a number of cytokines including TNF-α, IL-1β, PDGF, bFGF, and TGF-β. Conversely, other cytokines, such as IL-8, MCP-1 and osteopontin released by interstitial cells and smooth muscle cells are chemotactic for macrophages, and this together with an upregulation of adhesion molecules contribute to their localisation in areas of injury. This perpetuates and amplifies the fibrogenic signals.

Cytokines and growth factors

Cytokines and growth factors play an important role in the chronic phase. They have profound effects on cells of the graft and on the immune system. Cytokines and growth factors are pleiotropic, with biological effects on many cell subpopulations, are regulated via autocrine, paracrine or systemic pathways, and there is a great deal of redundancy in the cytokine networks. The advent of the transgenic and knock-out technology has allowed to dissect out molecular pathways causally involved in allograft arteriosclerosis.[238,239] No cytokine has been unequivocally identified as specific to alloimmune response.

The redundancy of the cytokine system has been stressed by gene knockout technology: IL-4 is not necessary for the development of graft coronary arteriosclerosis, nor does its absence appear to augment the development of vascular lesions. In addition, TNF-αR_I deficiency in either donor heart or recipient does not abrogate the development of graft arteriosclerosis.[240] The

increased expression of TGF-β_1 has been linked to transplant arteriosclerosis both from clinical and experimental studies, and transfection of TGF-β to the kidney led to increased accumulation of the extracellular matrix and glomerulosclerosis.[241] Interestingly, cardiac allografts in TGF-β_1 deficient recipients developed significantly more intimal hyperplasia than controls.[242] In 1989, IFN-γ has already been postulated by Libby *et al.* to play a central role in CTD because of its effects on T cells and macrophages, as depicted above.[243] The availabilty of IFN-γ deficient mice permitted this group to test critically the contribution of IFN-γ to the development of CTD.[244,245] Cardiac allografts in IFN-γ deficient mice developed only minimal or no transplant arteriosclerosis as compared to controls. In addition, similar results on graft arteriosclerosis were -found after the administration of IFN-γ neutralizing antibody in normal rats.

1.4.3. Chronic remodelling

The process eventually becomes irreversible, but the period in which this occurs is variable: Retransplantation of allogeneic kidney grafts back into the original donor strain prevents CTD, when the retransplant is performed within 12 weeks, but not after this period.[246] In aorta and cardiac allografts Mennander *et al.* and Izutani *et al.* reported a much shorter time interval after which intimal hyperplasia continues, when the graft was transplanted back into the donor strain.[247,248]

Smooth muscle cells

Once the endothelial cells are injured, the secreted cytokines, i.e. IL-1, PDGF, IGF-1, TGF-β and bFGF, and metabolic products, such as prostaglandin, nitric oxide, and oxidized low-density lipoproteins induce smooth muscle cell (SMC) proliferation, as reviewed by Ross.[249] Activated T cells and macrophages, often in close anatomical association with the replicating SMC, produce also a whole wealth of these factors. Platelets deposited along the injured vascular wall contribute by secreting PDGF, EGF, TGF-β and tromboxane-A_2. When the SMC migrates to the intima, they transform their phenotype from 'contractile' to 'secretory' and the cells become capable of replication.[249,250] In addition, SMC can produce many of these growth factors and may generate similar autocrine or paracrine loops of stimulation for cell replication, as seen in 'classical' atherosclerosis.[249] These factors also may modulate extracellular matrix synthesis, angiogenesis, and leucocyt adhesion. Moreover, activated SMC can

express MHC class I and II and may act as antigen presenting cells.
Numerous drugs inhibit SMC proliferation, and some have been shown to be of some benefit in organ allografts, such as angiopeptin.[251-253]

Extracellular matrix
As the endothelium is damaged, the underlying extracellular matrix can become activated and act as costimulator for leucocytes to facilitate recruitment and extravasation. For instance, exposed collagens and fibronectin may act as costimulators for activated $CD4^+$ T-cells.[254,255] After antigen activation naïve T cells synthesize heparanase, which facilitates migration through tissue.[256] The cleavage of heparan sulphate by this enzyme also activates and releases fibrogenic growth factors, such as basic fibroblast growth factor in the extracellular matrix.[257] TGF-β, produced by the activated T cells and macrophages, stimulates the production of ECM molecules and inhibits the matrix degrading enzymes. The thickening of basal membranes, such as that of the pericapillary and glomerular endothelium in the transplanted kidney, interstitial fibrosis and sclerosis, and in intima hyperplasia smooth muscle cell proliferation is accompanied by excessive synthesis of connective tissue proteins.

1.5 Conclusion

CTD is currently the main cause of late graft failure. CTD is usually associated with previous acute rejection episodes, although several non-alloantigen associated factors, like ischemia, hyperlipidemia, and hypertension may enhance the process. We propose that the process leading to CTD in allografts begins at time of graft retrieval, is enhanced by ischemic injury, which provokes an alloimmune response to the endothelial cells, the extracellular matrix and parenchyma. An ongoing alloimmune response in which several non-alloimmune factors may interfere, eventually leads to irreversible lesions of the organ graft.

1.6 Aim of the thesis

Chronic transplant dysfunction (CTD) hampers indefinite survival of solid organ grafts, such as kidney, heart and lung. Long-term survival of clinical intestinal transplants, which survival until recently was too short to be encountered by CTD, appears to be hampered by CTD also. There is still no way to prevent CTD and a rational treatment is not within hand's reach, since its etiology and pathophysiology are poorly known. At present, retransplantation is the only effective therapy.

Clinical and experimental studies indicate that alloantigen-dependent events play an important role in the development of CTD. Nonetheless, the contribution of non-alloantigen specific factors, such as ischemia, has also been suggested.

Prospective clinical studies to unravel aspects of CTD cannot easily be realized due to the contribution of multiple variables, need of a large patient population,and a long follow-up period. From a mechanistic point of view, clinical studies cannot discriminate whether an effect of non-alloantigen specific factors acts directly or via potentiating alloimmune responses, since allogenicity is a *conditio-sine-qua-non* in the clinical setting. To overcome these difficulties, we investigated some etiological or pathophysiological aspects of CTD in transplantation models in the rat, with the ultimate goal to obtain new therapeutic strategies.

We investigated the role of non-alloantigen specific factors in the development of CTD. At first, we studied the effect of surgical trauma and prolonged cold ischemia on the development of transplant arteriosclerosis *per se*, the cardinal histologic feature of CTD, using the well-established aorta transplantation model.

Subsequently, the significance of prolonged cold ischemia on the development of CTD was verified in a solid organ transplant, - i.e. the vulnerability of intraparenchymal vessels and parenchymal tissue to ischemia and to understand a possible relationship between arteriosclerosis and parenchymal lesions. Therefore, we introduced a kidney transplantation model of CTD into our laboratory.

Using this model, the effect of prolonged cold ischemia on the development of CTD was studied in both kidney isografts and allografts, treated transiently with the immunosuppressive agent Cyclosporin. Analysis of infiltrating cells at different timepoints during the course gives us insight into the mechanism.

Without use of any immunosuppressive drug, a relationship between ischemia and allogenicity in kidney allografts was investigated.

In kidney transplantation, complications of urinary tract reconstruction can lead to late graft failure. We compared long-term graft function in kidney transplants with 2 different surgical techniques of urinary tract reconstruction applied: uretero-ureterostomy versus uretero-neocystostomy.

Genes other than MHC-genes may influence the development of CTD. Some transplant studies suggest differences in graft outcome due to donor ethnicity. To elucidate whether the descent plays a role, the impact of genetic background of donor kidneys on CTD was studied in a syngeneic environment.

Since we had previously shown that CTD occurs in intestinal transplants a rat model was set up to study the pathophysiology of CTD in small bowel transplants. Functional, histologic and inflammatory features of CTD in this model were characterized. This model was subsequently used to investigate growth factor patterns.

Gene expression of EGF and TGF-β_1 in the small bowel transplants with CTD was investigated.

The gene and protein expression of bFGF and its localization was studied in the small bowel transplants.

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The role of cold ischemia and surgical trauma on transplant arteriosclerosis

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Summary

Background: Late graft loss is predominantly attributable to chronic transplant dysfunction (CTD). The general histological feature of CTD is transplant arteriosclerosis. Although alloantigen-dependent factors are central in the development of CTD, it is still a matter of debate whether alloantigen-independent factors contribute to this process as well.
Aim: The aim of this study was to investigate the role of two factors, namely 1) cold ischemia and 2) surgical trauma on the development of transplant arteriosclerosis in a rat aorta transplantation model.
Materials and methods: Aorta grafts (10 mm length) were transplanted orthotopically; syngeneic transplantations were performed between inbred BN rats, and allogeneic transplantations from WAG to BN rats. The grafts were stored at 4 °C in UW-solution, and two cold ischemia times were applied: 0.1 and 24 hours. To investigate the impact of surgical trauma grafts were anastomosed with continuous or interrupted sutures, or were implanted non-stretched versus stretched. The stretch model was performed by transplanting 8 mm aorta segments into 12 mm dissected recipients. Inversely, 12 mm segments were transplanted into the 8 mm recipients to assure non-stretch. The rats were sacrificed 4 weeks post-transplantation Histomorphological analysis from the midportion of aorta grafts consisted of measurement of intimal thickness, medial thickness and nuclear density, and adventitial nuclear density.
Results: Syngeneic grafts preserved for 24 hours had significantly less intimal hyperplasia and a significantly higher adventitial nuclear density than aorta isografts with 0.1 hr cold ischemia (16±5 vs. 39±7 μm, and 113±2 vs. 169±7 nuclei/0.1 mm^2, respectively). In allografts, results of all studied parameters did not differ between the 0.1hr and 24hr cold ischemia-groups. Continuous suturing enhanced intimal thickening significantly compared to interrupted suturing (39±7 vs. 14±6 μm, respectively), whereas stretch did not influence intimal hyperplasia. On the other hand, aortic stretch significantly reduced medial thickness and nuclear density compared to non-stretched aortic grafts (83±2 vs. 94±2 μm and 149±7 vs. 199±9 nuclei/0.1 mm^2, respectively).
Conclusions: These results indicate that alloantigen-independent factors contribute to CTD: 1) Cold ischemia up to 24 hours does not enhance transplant arteriosclerosis. 2) Continuous suturing of the vessel aggravates intimal hyperplasia and 3) vascular stretch decreases medial thickness.

Introduction

One year allograft and patient survival continue to improve, principally due to progress in immunosuppressive therapy. Nonetheless, from one-year post-transplantation, there is a steady rate of organ failure due to chronic transplant dysfunction (CTD), a comprehensive term used to denote several pathological events that lead to a gradual, irreversible deterioration of organ function.[1] The cardinal histopathological feature of CTD in all organ transplants is transplant arteriosclerosis,[2,3] characterised by concentric thickening of the intima, migration and proliferation of smooth muscle cells, medial necrosis and adventitial inflammatory infiltrate.

Some clinical studies showed that prolonged cold ischemia is a risk factor,[4,5] whereas others did not.[6,7] Experimental studies of organ transplantation are also inconsistent with regard to the role of cold ischemia on CTD.[8,9] Also, the effects of surgery per se are not uniform; One study showed that syngeneic kidney transplantation led to a fall of glomerular filtration rate compared to unilaterally nephrectomized rats.[10] On the other hand, Tullius *et al.* could not demonstrate adverse effects of manipulating the kidney: clamping, dissecting, dividing and consecutively reanastomosing the renal vessels or ureter did not lead to functional or histological changes of the kidney.[11]

The aim of this study was to elucidate the influence of these two factors, namely cold ischemia and surgical trauma, on the development of transplant arteriosclerosis in a rat aorta transplantation model. To exclude organ specific factors on the development of CTD the aorta graft was used, which is a proven CTD model.[12] To study these factors in the absence of allogenicity, syngeneic grafts were compared with allogeneic ones.

Materials and methods

Animals

Adult male Brown Norway (BN) and Wistar-Agouti (WAG) rats weighing 250-275 g were used. These rat strains differ at the major histocompatibility locus: The BN is homozygous for the RT-1^n haplotype, whereas the WAG strain is homozygous for RT-1^u haplotype. The animals were purchased from Harlan-CPB (Austerlitz, The Netherlands), where they were bred under specific

pathogen free conditions. 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 and adhered to the rules laid down in the published "Guidelines on the protection of experimental animals' by the Council of the EC (1986).

Aorta transplantation

All rats were anaesthetised with ether whereafter a laparotomy was performed. In the donor a segment of infrarenal aorta (10 mm length) was isolated, excised and perfused with University of Wisconsin (UW) solution (Viaspan®, Du Pont Pharmaca) of 4°C. The graft was preserved for 0.1 hr, i.e. the minimal cold ischemic period possible with our transplant procedure. In the recipient, the aorta was transplanted orthotopically. End-to-end anastomosis was performed using a 9.0 monofilament nylon suture (B.Braun, Neuhausen, Germany). The perioperative warm ischemic time was 20 minutes.

Experimental groups

Syngeneic transplantations were performed between BN rats, and allogeneic transplantations from WAG to BN rats. No immunosuppressive therapy was given.

To study the impact of cold ischemia on graft arteriosclerosis in both an allogeneic and syngeneic setting, the grafts were stored in UW-solution at 4°C for 0.1hr (BN-BN, n=6, WAG-BN, n=5) or 24hr (BN-BN, n=4, WAG-BN, n=4). The anastomosis was made with continuous sutures.

The impact of surgical trauma was studied in isografts by comparing the mode of suturing, continuous (n=6) or interrupted (n=5), and by comparing the way of implantation, stretched (n=5) versus non-stretched (n=5). The stretch model was performed by transplanting 8 mm aorta segments into 12 mm dissected recipients. Inversely, 12 mm segments were transplanted into the 8 mm recipients to assure non-stretch. Aortic transplants were removed at 4 weeks post-transplantation, since we previously demonstrated that in the WAG-BN model, intimal hyperplasia could not be evaluated beyond 4 weeks due to intense intimal thickening, thrombosis and necrosis.[12]

Histology

Formaldehyde-fixed and paraffin-embedded 5 μm-thick straight cross-sections from the midportion of the aorta graft were stained with hematoxylin/eosin and elastic of Gieson. The following variables were analysed using a calibrated ocular micrometer: Intimal thickness, medial thickness and nuclear density, and adventitial nuclear density. The average medial thickness and maximal intimal thickness were determined. Cellularity of the media and adventitia was assessed by counting the number of nuclei at 5 locations using a magnification of 400X. The mean score was multiplied to a field of 0.1 mm^2.

For statistical analysis, one way ANOVA followed by unpaired t test was used. The results are expressed as mean ± SD in the text and tables, and as mean ± SEM in the figures.

Results

Cold ischemia

Syngeneic aorta transplants with the minimal cold ischemic time of 0.1 hr developed some intimal hyperplasia, although much less than allogeneic grafts preserved for 0.1 hr (Figures 1a and b). In BN isografts preserved for 24 hrs intimal hyperplasia was significantly less compared to isografts with 0.1hr cold ischemic time. Medial thickness and nuclear density were similar in both

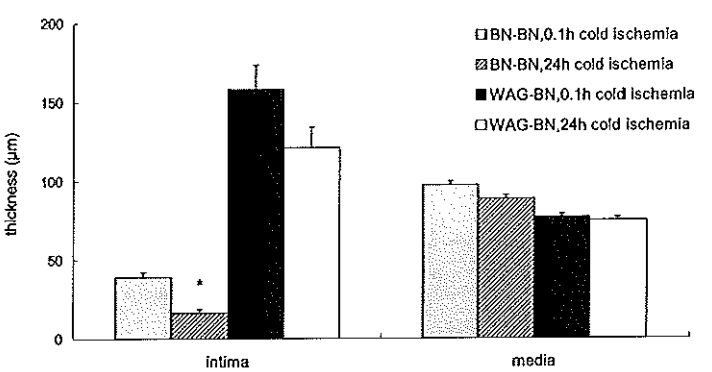

Figure 1A. The effect of 24 hr cold ischemia on intimal and medial thickness in aortic isografts and allografts. * = p<0.01 vs. BN-BN isografts with 0.1 hr cold ischemia

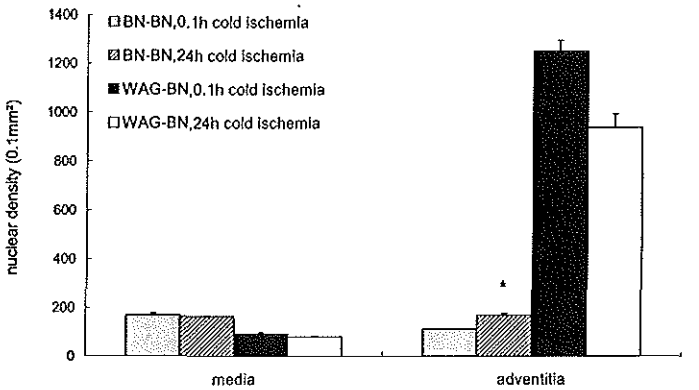

Figure 1b. Medial and adventitial nuclear density. * = p<0.05 vs. BN-BN isografts with 0.1 hr cold ischemia.

groups, whereas the isografts with 24 hr ischemia had significantly more adventitial cellular infiltration. In allografts, prolonged cold storage of the aorta for 24 hrs did not lead to significantly more histopathological changes than in aortic allografts preserved for 0.1 hr: The intimal thickness, the medial nuclear density and the cellular adventitial infiltration did not differ both groups.

Mode of suturing

In the BN-isografts, continuous suturing of the aortic anastomosis enhanced intimal thickening significantly compared to interrupted suturing. Medial and adventitial nuclear density was similar in both groups (Figure 2).

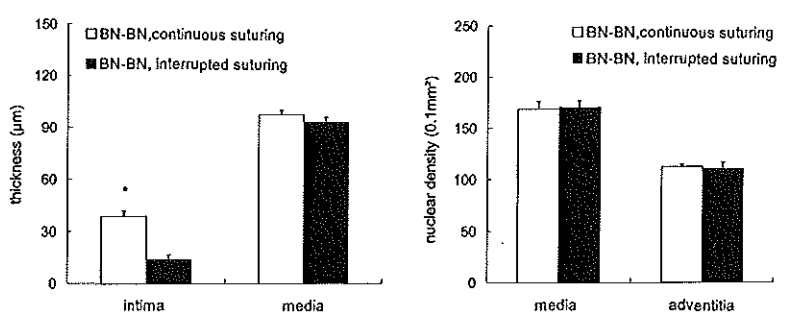

Figure 2 The effect of mode of suturing on intima, media and adventitia in aortic isografts. * = p<0.05 vs. BN-isografts, continous suturing

Mode of implantation

Implanting the aorta under stretch did not influence intimal hyperplasia, but significantly reduced medial nuclear density and led to a significant increase of the adventitial nuclear density (Table 1).

Group	intimal thickness (μm)	medial thickness (μm)	medial nuclear density(0.1mm²)	adventitial nuclear density (0.1 mm²)
BN-BN non-stretch	16 ± 6	94 ± 2	199 ± 20	90 ± 7
BN-BN stretch	10 ± 7	83 ± 2 *	149 ± 15 *	116 ± 18

Table 1. The impact of the mode of implantation on intima, media and adventitia in aortic isografts. * = p<0.05 vs. non-stretch.

Discussion

The etiology of CTD remains poorly understood. Since both alloantigen-dependent and independent factors, including ischemic time and surgical trauma, are thought to play a role, we examined these factors in syngeneic aorta grafts by varying the ischemic time, the mode of suturing and the degree of stretch. The effect of prolonged ischemic time was also studied in aortic allografts.

Firstly, the present study demonstrates that cold ischemia during 24 hours did not enhance transplant arteriosclerosis in both aortic iso- and allografts. This observation conflict with reports using also the aortic graft model in which prolonged ischemia produced vascular lesions in syngeneic transplants.[13,14] However these investigators preserved the aortas for 24 hr in cold saline[13] or, Frödin/Wolgast perfusion solution.[14] By using UW preservation solution, a superior preservation fluid which contains oxygen free radical scavengers as allopurinol and gluthatione, we found that cold ischemia had no adverse effect on the intimal thickening, suggesting that endothelial integrity was better preserved. On the other hand, the prolonged cold storage of the aorta led to a significant increase of infiltrating cells in the adventitia. Since studies show that adventitial infiltration precedes intimal thickening[12,15] and that the intensity of cellular adventitial infiltrates correlates with the degree of intimal hyperplasia,[12]

it is possible that the 28-day follow up was too short to demonstrate adverse effects of 24 hr preservation on intimal hyperplasia. Using the same rat strain, we recently demonstrated focal intimal thickening in the interlobular and cortical arteries in syngeneic kidney transplants preserved for 24 hr at 52 weeks post-transplantation.[16] The impact of cold ischemia in an allogeneic environment is also not clear. Similar to our findings, other investigators could also not demonstrate a significant effect of different cold ischemic times in aortic allografts.[14,17] On the other hand, these data conflict with findings using cardiac and renal allografts, in which prolonged cold ischemia led to more graft vasculopathy.[8,18] Comparison of different studies is complicated by strain and species differences, as well as different preservation solutions, immunosuppressive regimens and even dietary manipulation. Moreover, the response-to-injury in endothelium of aortas could be different from that of intraparenchymal arteries.

Secondly, the transplantation procedure *per se* did lead to some histological changes, since isografts with a minimal ischemic time showed intimal hyperplasia, which is absent in the native aorta. Results of the present study indicate that the mode of suturing influences the development of transplant arteriosclerosis. In the midportion of the 1 cm aortic transplant, interrupted suturing of the anastomosis led to significant less intimal thickening. Continuous suturing may lead to invisible torsion of the graft, thereby changing flow patterns. Decreased flow and flow separation causes decreased wall shear stress, which is known to provoke intimal hyperplasia.[19] Also, the way of implantation, i.e. the degree of stretch, influences transplant arteriosclerosis. Stretch did not have an impact on intimal thickness, but did significantly decrease the thickness of the media. Since mechanical stretch is known to induce apoptosis,[20] decreased nuclear density in the stretched aortic transplants could be the result of apoptosis.

Finally, the relationship between intimal thickness and decreased medial thickness, as a consequence of migration of smooth muscle cells, is very clear in allografts, but not in the different subjects presently studied in isografts. Mennander *et al.* also demonstrated that thickening of the intima was not accompanied by medial cell destruction in aortic isografts exposed to starch before transplantation, thereby suggesting that intima thickness and medial necrosis are independently regulated.[15] Therefore the response-to alloimmune, ischemia and mechanical-related-injury in the aorta appears to be mediated by different mechanisms.

In conclusion, 1) Cold ischemia during 24 hours using an optimal preservation solution does not enhance transplant arteriosclerosis in aortic grafts. 2) Continuous suturing of the vessel aggravates intimal hyperplasia, and vascular stretch decreases medial thickness.

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3

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

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Summary

Background: Chronic transplant dysfunction (CTD) is the major cause of late graft failure. In unravelling its etiology, non-alloantigen specific factors, as ischemia/reperfusion and renal mass have been suggested to play a role in the process.
Aim: To investigate the effect of the transplantation procedure itself on the development of CTD in a syngeneic kidney transplant model in the rat.
Materials and methods: Heterotopic kidney transplantation was performed with the BN rat as donor and recipient, in which the recipient kidneys were removed. Unilaterally nephrectomised (UNx) and native BN rats served as controls. Renal function was determined monthly (Proteinuria and Glomerular filtration rate/100gr body weight;GFR). The follow-up period was until 52 weeks post-transplantation. At sacrifice, the systemic blood pressure was measured. Histomorphological analysis of CTD according to the BANFF criteria was done. Immunohistochemical staining was performed to identify infiltrating cells (CD4, CD8, and ED-1) and the expression of MHC class II, and ICAM-1.
Results: Isografts had a minor, constant proteinuria during follow-up, which did not differ from that of UNx: 27±10 vs. 29±2 mg/24h at week 52. 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. The GFR of isografts did not differ from UNx rats at week 4 post-transplantation. The GFR in UNx and isografts did not further change during the next 48 weeks of follow-up. Histomorphology of renal isografts was comparable to UNx kidneys: some glomerulopathy and tubular atrophy, leading to a BANFF-score of 2.6±0.5 vs 2.7±0.8. Native kidneys demonstrated some glomerulopathy, resulting in a BANFF score which was similar to that of UNx. In native BN kidneys few $CD4^+$ cells and ED-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 localised in the glomeruli, and a more intense ICAM-1 expression in the glomeruli and interstitium.
Conclusion: Transplantation of one kidney in itself does not lead to CTD.

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 consistently at 7-8 year.[1] 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;[2] the chronic dysfunctioning kidney graft also shows less specific features as glomerular sclerosis, tubular atrophy and interstitial fibrosis. The mechanisms leading to CTD are largely unknown.[3,4] 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.[5]

Nonetheless, there are indications that alloantigen-independent factors can cause lesions resembling those seen in chronic rejected allografts. For instance, renal ischemia in unilaterally nephrectomised rats can cause late histomorphological lesions mirroring those seen in kidney allografts with CTD.[6,7] In syngeneic kidney transplants, Tullius *et al.* demonstrated late damage to the same extent as observed in ischemic non-transplant kidneys.[7] Others, however, did not see functional or morphologic changes in renal isografts.[8,9] The pathway from such early injury to the late morphologic changes is unclear. Since reduction of nephron number in non-transplant experiments can cause glomerular and vascular changes and thus resembling CTD,[10] 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 rats of inbred Brown Norway (BN; RT1^n) weighing 225-250g and 10-12 weeks old age 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.[11] The animals were anaesthetised 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 of 4 °C at a rate of 2 ml /min. The kidney was excised and stored in UW solution (4°C) for about 6 minutes 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. During surgery, the graft was wrapped in gauze moisturised with 4 °C phosphate buffered saline (PBS). The perioperative ischemia time was 30 minutes. After revascularization, the ureter was anastomosed end-to-end to the distal third part of the recipient's ureter using 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 nephrectomised (UNx) rats (n=8), and native non-nephrectomised rats (n=5). Functional, morphological and immunohistological evaluations were performed after engraftment.

Functional measurements

Urine was collected monthly by placing the rats individually for 24hr in metabolic cages. Protein excretion was measured colorimetrically by addition of pyrogallol red.[12] The glomerular filtration rate (GFR) per 100g body weight (BW) was based on the clearance of creatinine. Serum and urinary creatinine was determined using the Jaffé method without deproteinization.

At sacrifice, diastolic and systolic blood pressure (BP) was measured intra-arterially. After anaesthetising 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 minutes.

Histology

At 52 weeks post-transplantation, kidneys were harvested and weighed. They were fixed by immersion for 48h 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[1] by 2 independent investigators. Briefly, glomerulopathy, interstitial fibrosis, tubular atrophy and intimal thickening were separately determined with a score ranging from 0 = normal, 1 = up to 25% affected, 2= moderate, affecting 25-50%, and 3= more than 50% changes.

Immunohistology

Representative portions of all kidneys were stained on 5 µm cryostat sections by a three-layer immunoperoxidase technique. After fixation with acetone for 10 minutes, tissues were dehydrated through graded alcohols to block endogenous peroxidase activity by incubation for 10 minutes 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 one hour 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 each incubation, slides were washed in PBS-Tween 20, 0.1%. A second layer, rabbit anti-mouse IgG (Dako) was then applied for 30 minutes and after washing, slides were incubated with the third layer, mouse peroxidase-anti peroxidase (Dako) for 30 minutes. After washing in PBS, the reaction was developed by the addition of Diaminobenzidine substrate (Dako) and slides were counterstained in Mayer's hematoxylin for 40 sec, washed, dehydrated and mounted.

The analysis was done blindly as to 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 (Figure 1).

The GFR (per 100g 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 (Figure 2).

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

group	n	Diastolic BP	Systolic BP
BN	5	87 ± 5	117 ± 6
BN-UNx	8	86 ± 4	117 ± 3
BN-isografts	5	80 ± 4	115 ± 3

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

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±0.03g kidney/100g BW). Kidney transplantation led to the same degree of compensatory hypertrophy: Isografts had a relative kidney weight which did not differ from that of UNx-rats (0.48±0.04 vs 0.53±0.06g kidney/100g BW).

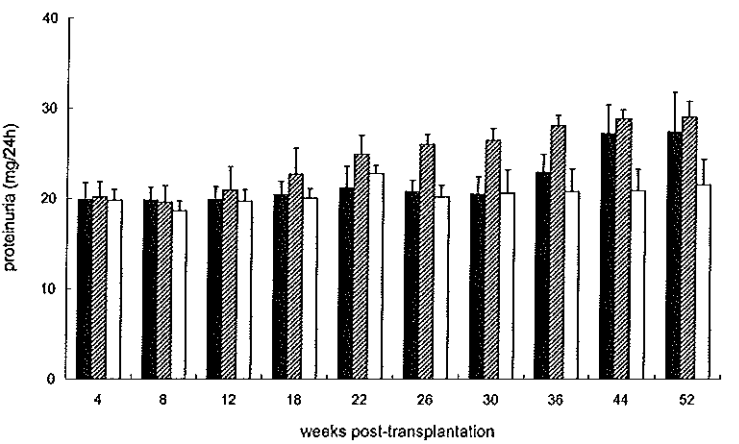

Figure 1. Proteinuria in renal isografts (■), unilaterally nephrectomized (▨), and native controls (□). Isografts had a stable proteinuria during follow up, and the excretion was similar to that of UNx rats.

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 was not seen in the controls. The total BANFF score was 1.5±0.6.

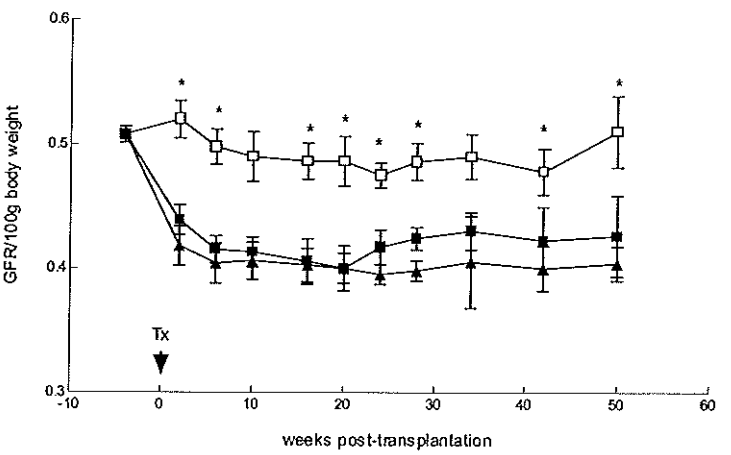

Figure 2. Relative glomerular filtration rate in renal isografts (■), unilaterally nephrectomized (UNx)(▲) and native controls (□). Isografts and UNx had similar GFRs, which were about 80% of that in native controls. * = $p<0.05$ vs UNx at the same timepoint; Tx = transplantation.

Immunohistology

Native BN-kidneys showed some $CD4^+$ cells and ED-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 the 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.

	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 macrophages	12 ±3 *	6 ±1	5 ±1
MHC class II	1.5	1.0	1.0
ICAM-1	2.0 *	1.0	1.0

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/0.1mm^2; MHC II and ICAM-1 expression were quantified on a 0-4 scale. *= $p<0.05$ vs. UNx and native controls.

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, proteinuria, GFR, and a systemic BP were similar in isografts and UNx rats. Renal morphology was comparable: minor glomerulosclerosis, some glomerulopathy and tubular atrophy, without interstitial fibrosis and intimal thickening, although the infiltration with $CD4^+$ cells and macrophages was significantly higher in isografts. These findings conflict with those 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.[7] These changes appeared to be induced mainly due to ischemia. Clamping the renal vessels of a unilaterally nephrectomised 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 did also not observe intimal thickening after syngeneic kidney transplantation using DA or AS rat strains,[8,9] 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; a phenomenon which has been demonstrated by Ibrahim *et al*: 60 minutes 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.[14]

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 was not associated with late renal injury. As renal injury in a hyperfiltrating kidney only develops when accompanied by glomerular hypertension, it is presumable that the hyperfiltrating BN-kidney preserved a normal glomerular pressure. Since UNx Lewis rats showed increased protein excretion, increased ICAM-1 and MHC class II expression at week 52,[7] 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.[15] Also, the age at time of nephrectomy and the dietary protein intake have been demonstrated to be important determinants for the lifespan of a single kidney.[16] In the light of the results of the present study, the suggestion that glomerular changes seen in human kidney isografts could be the result of the transplant procedure *per se* needs to be revised.

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

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4

Late graft dysfunction after prolonged cold ischemia of the donor kidney. Inhibition by Cyclosporine

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Transplantation, in press

Summary

Background: The present study was devised to elucidate the influence of prolonged cold ischemia on the development of chronic transplant dysfunction (CTD) in kidney isografts (BN-BN), and in kidney allografts (BN-WAG) under temporary Cyclosporine (CsA) therapy.
Methods: To induce ischemic injury, BN-donor kidneys were preserved for 24 hr in 4°C University of Wisconsin solution before transplantation. Renal function (proteinuria), histomorphology according to the BANFF criteria for CTD, and infiltrating cells were assessed. Grafts were examined both early at day 2, 3, 6, and 10, and late at week 26 (allografts) or at week 52 (isografts).
Results: Non-ischemic isografts preserved a normal function and morphology. Ischemic isografts developed a progressive proteinuria over time, and demonstrated significantly more glomerulopathy with macrophage (MΦ) infiltration and intimal hyperplasia than non-ischemic controls at week 52. During the initial 10 days, there was an increased infiltration of MHC class II$^+$ cells, predominantly CD4$^+$ cells and MΦ, coinciding with upregulated ICAM-1 expression. CsA-treatment in ischemic isografts inhibited infiltration of MHC II+ cells in the early stage which was accompanied by significantly less renal damage at week 52 compared to untreated controls (proteinuria: 59±8 vs 134±19mg/24h; BANFF score: 2.8±0.4 vs 4.3±1.0).
Under CsA therapy, 24h cold ischemia of the allograft did neither affect the onset or progress of proteinuria, nor the histomorphology (BANFF score: 7.8±2.4 vs 7.3±1.9). In both ischemic and non-ischemic allografts, ICAM-1 expression and mononuclear cell infiltration (CD4, CD8, MΦ) was abundantly present during the first 10 days, and function deteriorated rapidly.
Conclusions: Prolonged cold ischemia plays a role in the induction of CTD, but its deleterious effect can be successfully inhibited by CsA. Therefore, the alloantigeneic stimulus is the overriding component in the multifactorial pathogenesis of CTD.

Introduction

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;[1] the chronic dysfunctioning kidney graft also shows less specific features as glomerular sclerosis, tubular atrophy and interstitial fibrosis. The mechanisms leading to CTD are unknown.[2,3] Clinical data suggest that the host alloimmune responsiveness plays an important role in the development of CTD: Acute rejection is the most consistent risk factor for CTD.[4] Nonetheless, there are indications that ischemia/reperfusion injury inducing a non-alloantigen specific response, could be implicated in the pathogenesis also: The United Network for Organ Sharing as well as some single centres reported that prolonged cold ischemia of the cadaveric kidney shortens late graft survival.[5-7] Moreover, the superior late graft survival rates of living-donor transplants, both related and unrelated compared to that of cadaveric kidneys could reflect diminished cold ischemia/reperfusion injury.[8]

However, it is not clear whether cold ischemia *per se* is a risk factor, or only in an interplay with other pathogenic factors.[6] Experimental non-transplant studies showed that renal ischemia in itself causes late lesions resembling those seen in allografts with CTD.[9] The impact of cold ischemia on kidney CTD or on other organ transplants in an allogeneic environment is less obvious.[10-12] Comparison of these studies are hindered by a variety of used preservation solutions, storage methods, type of immunosuppressive therapy in the initial phase, as well as donor status.

Therefore, the objectives of the present study were to investigate the effect of prolonged cold ischemia of a donor kidney on the development of CTD in a syngeneic and in an allogeneic setting. To prevent acute rejection in the allografts, Cyclosporine A was administered.

Materials and methods

Animals

Male rats of the inbred Brown Norway (BN)(RTI^{n}) and WAG/Rij (RTI^{u}) strains, were purchased from Harlan, Austerlitz, The Netherlands. The rats weighed 200-250 g and were 2-3 months of age at the beginning of the experiment. 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

Transplantations were performed using a modification of the technique described by Fisher and Lee.[13] The animals were anaesthetised 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 of 4 °C at a rate of 2 ml /min. The kidney was excised and stored in UW solution 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 (B Braun, Melsungen, Germany). During surgery, the graft was wrapped in a gauze moisturised with 4 °C phosphate buffered saline (PBS). The perioperative ischemic time was 30 minutes. After revascularization, the ureter was anastomosed end-to-end to the distal third part of the recipient's ureter using interrupted 10-0 or 11-0 prolene sutures. The left, native kidney was removed at time of transplantation, whereas the contralateral one was excised 3 weeks later.

Cold ischemia period

The donor kidney was stored at 4 °C for either 10 minutes, i.e. the minimal time in which the recipient is anaesthetised and prepared for implantation of the graft, or 24 hours. The 10 minutes cold ischemia will subsequently be referred to as non-ischemia.

Immunosuppressive regimen

Immunosuppression consisted of intramuscular Cyclosporine A (CsA; Novartis, Basel, Switzerland) at a dosage of 10 mg/kg at day 0, 1, 4 and 7 and subsequently at a dose of 5 mg/kg, 3 times a week for 2 weeks. In a pilot-study this CsA schedule was able to overcome acute rejection in allografts and allowed for the development of CTD. If untreated, BN-WAG allografts develop a fatal acute rejection in 8-10 days.[14]

Experimental design

The experimental groups are enumerated in Table 1. The effect of 24 hours of cold ischemia on the BN donor kidney, was studied in BN and WAG recipients. The effect of cold ischemia in BN-BN isografts (group 2) was compared to non-ischemic isografts (group 1). Simulating the situation that occurs during clinical kidney transplantation, the effect of 24 hr cold ischemia was also studied in an allogeneic environment. WAG animals in group 6 received BN allografts that were subjected to 24 hr cold ischemia and compared to non-ischemic allografts (group 5). To reverse acute rejection in these allografts, a short course of CsA was administered.

To compare the effect of cold ischemia between isografts and allografts, CsA was given to isografts, with and without cold ischemia (groups 3 and 4).

Group	Strain combination	Cold ischemia pre-kidney Tx	Cyclosporine	Organ removal (days)	(weeks)
1	BN→BN	-	no	2,3,6,10	52
2	BN→BN	24 hr	no	2,3,6,10	52
3	BN→BN	-	yes	2,3,6,10	52
4	BN→BN	24 hr	yes	2,3,6,10	52
5	BN→WAG	-	yes	2,3,6,10	52
6	BN→WAG	24 hr	yes	2,3,6,10	52

Table 1. Experimental design

Morphological and immunohistological evaluations of kidney specimens of all groups were performed at day 2, 3, 6, and 10 post-transplantation (n=3 animals/group/timepoint). For functional, late morphological and immunohistological evaluations, the isograft-groups were followed until week 52 after transplantation (Isografts: n=5; Isografts, 24 hr cold ischemia: n=12; Isografts, CsA: n=5; Isografts, 24 hr cold ischemia, CsA: n=5). The allograft-groups were sacrificed at week 26 because of end-stage graft failure. (Allograft, CsA: n=12; Allograft, 24 hr cold ischemia, CsA: n=7).

Functional studies

Urine was collected monthly by placing the rats individually during 24 hr in metabolic cages. Protein excretion was measured colorimetrically by addition of pyrogallol red.[15] Serum creatinine was determined using the Jaffé method without deproteinization.

Histology

Kidneys were harvested under ether anesthesia, fixed by immersion for 48h 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 and Goldner and were evaluated for chronic transplant dysfunction according to the BANFF-criteria[16] by 2 independent investigators (E.A.K and U.H.). Briefly, glomerulopathy, interstitial fibrosis, tubular atrophy and intimal hyperplasia were separately determined with a score ranging from 0 = normal, 1 = up to 25% affected, 2= moderate, affecting 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 5 μm cryostat sections by a three-layer immunoperoxidase technique. After fixation with acetone for 10 minutes, endogenous peroxidase activity was blocked by incubation for 10 minutes in methanol/0.03% H_2O_2, after dehydration through graded alcohols. 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 one hour 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 each incubation, slides were washed in PBS-Tween 20, 0.1%. A second layer, rabbit anti-mouse IgG (Dako) was then applied for 30 minutes and after washing, slides were incubated with the third layer, mouse peroxidase-anti peroxidase (Dako) for 30 minutes. After washing in PBS, the reaction was developed by the addition of Diaminobenzidine substrate (Dako) and slides were counterstained in Mayer's hematoxylin for 40 sec, washed, dehydrated and mounted.

The analysis was done blindly as to the experimental group. Positive cells were counted at 400 x magnification using a calibrated micro-ocular grid in >16 fields

of view and expressed as number of positive cells /0.1 mm^2. The intensity of ICAM-1 expression on endothelium and tubules was quantified on a 0-to-3 scale (0 = none, 1 = mild, 2 = moderate, 3 = dense). Expression of ICAM-1 on infiltrating cells was not involved in the grading.

Statistical analysis

Statistical analysis was performed using Kruskal-Wallis one way ANOVA followed by Mann-Whitney test for the ordinal data from histology and ICAM-1 expression. One way ANOVA followed by a NewMan-Keuls test was used for the data from function and immunohistology. Statistical significance was established at $p<0.05$. The results are expressed as mean ± SD in the text and tables, and as mean ± SEM in the figures.

Results

Function

Cold ischemia in Isografts

Recipients of a non-ischemic isograft had a stable protein excretion during the whole observation period of 52 weeks (Figure 1A). 24 hr cold ischemia led to a significant, progressive increase of proteinuria from week 18 onwards compared to non-ischemic isografts. By 52 weeks, protein excretion had increased to 134±66 mg/24h versus 27±10mg/24h ($p=0.005$). CsA treatment in non-ischemic isografts led to a minor, but significant increase of proteinuria ($p=0.03$). Interestingly, CsA therapy in ischemic isografts led to significantly less proteinuria compared to the untreated controls (Figure 1A).

group ↓ / week →	4	8	12	18	26	36	44	52
Isograft	67±3	67±4	65±5	63±5	64±4	63±4	66±4	65±6
Isograft, ischemia	86±19*	72±9	68±9	69±6	71±5	68±6	73±12	85±19*
Isograft,ischemia,CsA	79±3	68±1	68±4	67±3	64±5	70±2	76±8	77±11
Isograft,CsA	80±7 †	73±9	67±2	63±4	62±6	67±2	67±6	68±5

Table 2A. Serum creatinine levels(µmol/l) in non-ischemic and ischemic kidney isografts, with and without Cyclosporine A treatment. * =$p<0.02$ vs Isograft; † =$p<0.05$ vs Isograft.

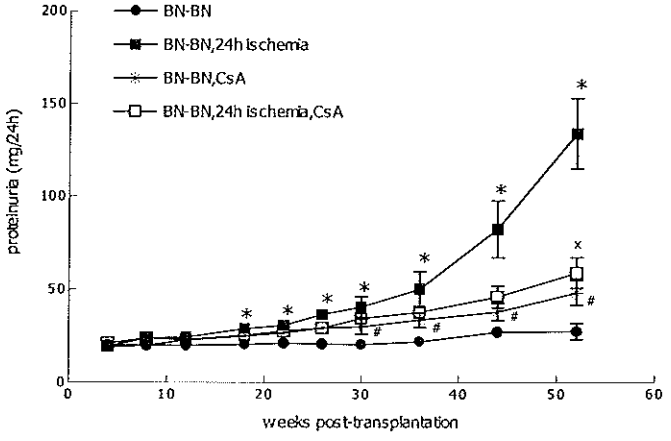

Figure 1A. Urinary protein excretion in ischemic and non-ischemic kidney isografts, with and without Cyclosporine therapy. Protein excretion of isografts remained at baseline level during follow-up. Isografts with 24h cold ischemia developed proteinuria from week 18, which increased progressively thereafter. (* =p<0.05 vs BN-BN). Cyclosporine A therapy in 24 hr isografts led to significantly less proteinuria compared to the untreated ones at week 52. (X =p<0.05 vs BN-BN,24h ischemia), whereas this therapy in non-ischemic kidneys led to a small, but significant increase.

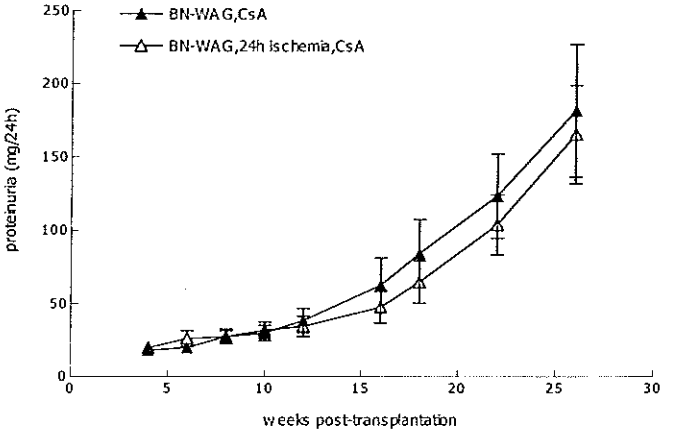

Figure 1B. Urinary protein excretion in ischemic and non-ischemic kidney allografts. From week 10 protein excretion increased progressively. Exposure of the donor kidney to 24h cold ischemia did not affect the onset or progression of protein loss in an allogeneic environment.

As shown in Table 2A, ischemic isografts and CsA treated isografts had both a significantly higher serum creatinine level at the first measurement, 4 weeks after transplantation than their controls. Thereafter, levels were comparable in the 4 groups until week 44. Than, the serum creatinine again increased in rats with an ischemic kidney isograft.

Cold ischemia in Allografts

Rats with a non-ischemic kidney allograft developed obvious proteinuria by week 10 and excreted progressively increasing amounts thereafter (Figure 1B). At week 22, some rats in this group were found to have uremia as consequence of end stage graft failure. Therefore, all animals were sacrificed at week 26, although 3 out of 12 rats had only a slightly increased protein excretion at this timepoint. Prolonged cold ischemia (24 hr) in the allografts did not affect the onset or progression of proteinuria compared to the non-ischemic allografts (Figure 1B): at week 26, the protein excretion was 165±82 vs 182±57 mg/24hr.
As in the syngeneic setting, ischemic allografts had a higher serum creatinine at 4 weeks post-transplantation compared to the non-ischemic controls (Table 2B). Thereafter, levels were comparable.

group ↓ / week →	4	8	12	18	22	26
Allograft,CsA	80±18	68±9	67±10	67±11	81±34	96±57
Allograft, ischemia,CsA	99±18*	80±18	80±16	83±23	92±25	109±30

Table 2B. Serum creatinine levels (μmol/l) in kidney allografts subjected to cold ischemia under Cyclosporine A therapy. * =p<0.05 vs Allograft,CsA.

Histology

Cold ischemia in Isografts

Exposure of the donor kidney to 24 hr of cold ischemia led to swelling of the endothelial cells of intraparenchymal arteries, and to tubular necrosis with oedema of the interstitium. Moreover, tubule cells contained lysosomal enzymes and the tubules were filled with protein casts at day 2 and 3 after transplantation, which was not seen in the non-ischemic isografts. After one year, these ischemic

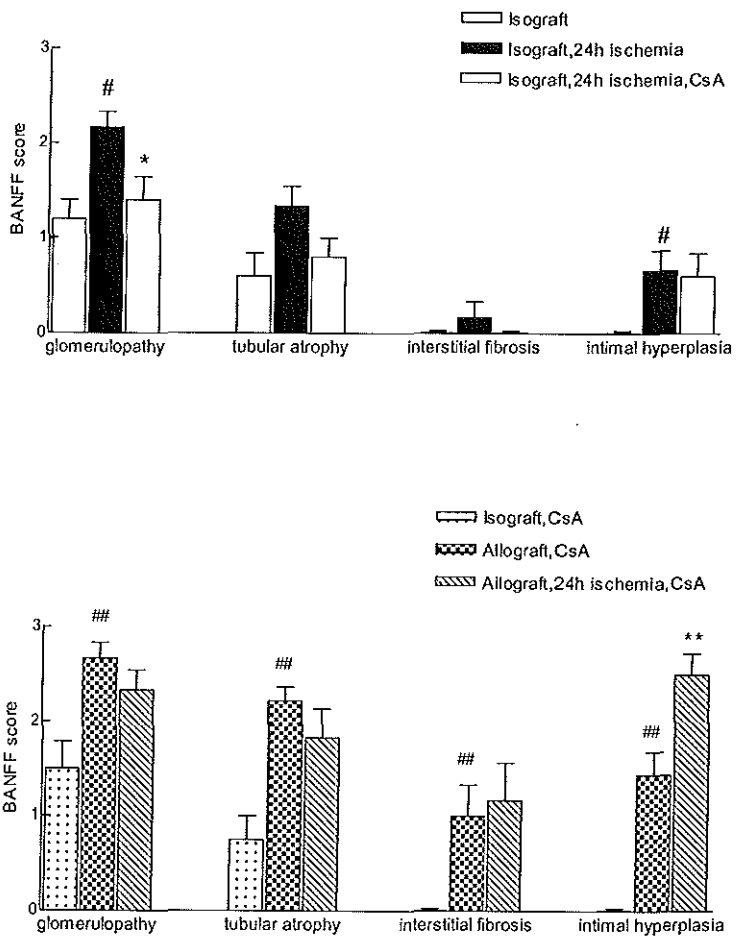

Figure 2. Histologic CTD in kidney iso- and allografts subjected to 24h cold ischemia. Isografts preserved for 24h demonstrated more glomerulopathy and intimal hyperplasia than non-ischemic isografts at 52 weeks post-transplantation (# = $p<0.05$). CsA-therapy in the ischemic isografts inhibited the development of glomerular lesions versus untreated ones (* = $p<0.05$), whereas it did not affect the degree of tubular atrophy or intimal hyperplasia.
CsA immunosuppressed kidney allografts subjected to 24h cold ischemia demonstrated more intimal hyperplasia than non-ischemic allografts at 26 weeks post-transplantation. (** = $p<0.05$). The allografts showed all signs of CTD moderately, whereas isografts under the same immunosuppressive regimen showed only minimal glomerulopathy and tubular atrophy (## = $p<0.05$).

isografts had more glomerulopathy (p=0.01) and intimal hyperplasia (p=0.03) than non-ischemic controls (Figure 2): the total BANFF scores were 4.3±1.0 and 1.8±0.4, respectively (p=0.004). By week 52, CsA-treatment in ischemic isografts resulted in less glomerulopathy, but did not affect the degree of intimal hyperplasia. The total BANFF score was significantly lower than that in untreated ones: 2.8±0.4 the vs 4.3±1.0 (p=0.01).

Cold ischemia in Allografts

At week 26, both ischemic and non-ischemic allografts demonstrated the histologic characteristics of renal CTD. Apart from more prominent perivascular infiltrates and intimal hyperplasia, prolonged cold ischemia did not affect the severity of the parenchymal lesions (total BANFF score 7.8±2.4 vs 7.3±1.9 in non-ischemic allografts).

Immunohistology

Cold ischemia in Isografts

The type and number of infiltrating cells is depicted in Figure 3, adhesion molecule expression in Figure 4. Significantly higher numbers of $CD45^+$-leucocytes had infiltrated the interstitium of ischemic isografts from day 2 compared to non-ischemic controls. The number of MHC class II^+ cells were higher, representing $CD4^+$ cells and $ED1^+$ macrophages. $CD8^+$ lymphocytes did not appear to play a role in ischemic injury. A further increase of these infiltrating cells coincided with increased expression of ICAM-1 in the peritubular capillaries at day 3. Thereafter the cell populations remained stable until day 10. One year post-transplant, the ischemic isografts still showed significantly more $CD4^+$ cells and $ED1^+$ macrophages, with a considerable number present in the glomeruli.

Whereas CsA treatment did not affect number and pattern of infiltrating cells in non-ischemic isografts, it inhibited cell infiltration of ischemic isografts (Figures 3A and B). The number of MHC class II^+, $CD4^+$ cells and macrophages were lower compared to non-treated controls (Figure 3A). One year post-transplant, they had significantly less $ED1^+$ macrophages, predominantly as consequence of a virtual absence of these cells in the glomeruli (2.4± 0.6 vs 7.8±1.2 cells/glomerulus).

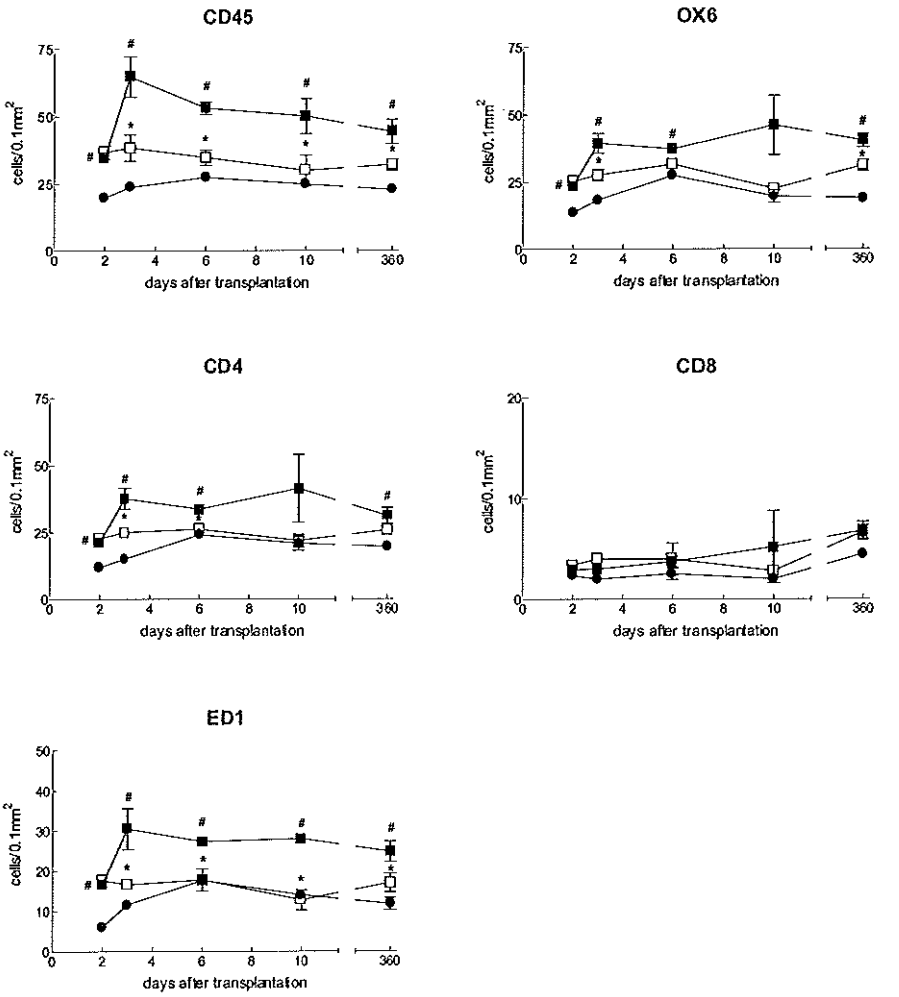

Figure 3A. Cell infiltration into 24 hr ischemic kidney isografts (■) is increased from day 2 onwards compared to non-ischemic controls (●), except $CD8^+$ lymphocytes. Treatment with CsA in ischemic isografts (□) inhibits this early influx. CD45 = pan leucocytes, OX-6= MHC class II. # =$p<0.05$ vs isograft; *= $p<0.05$ vs isograft,24h ischemia

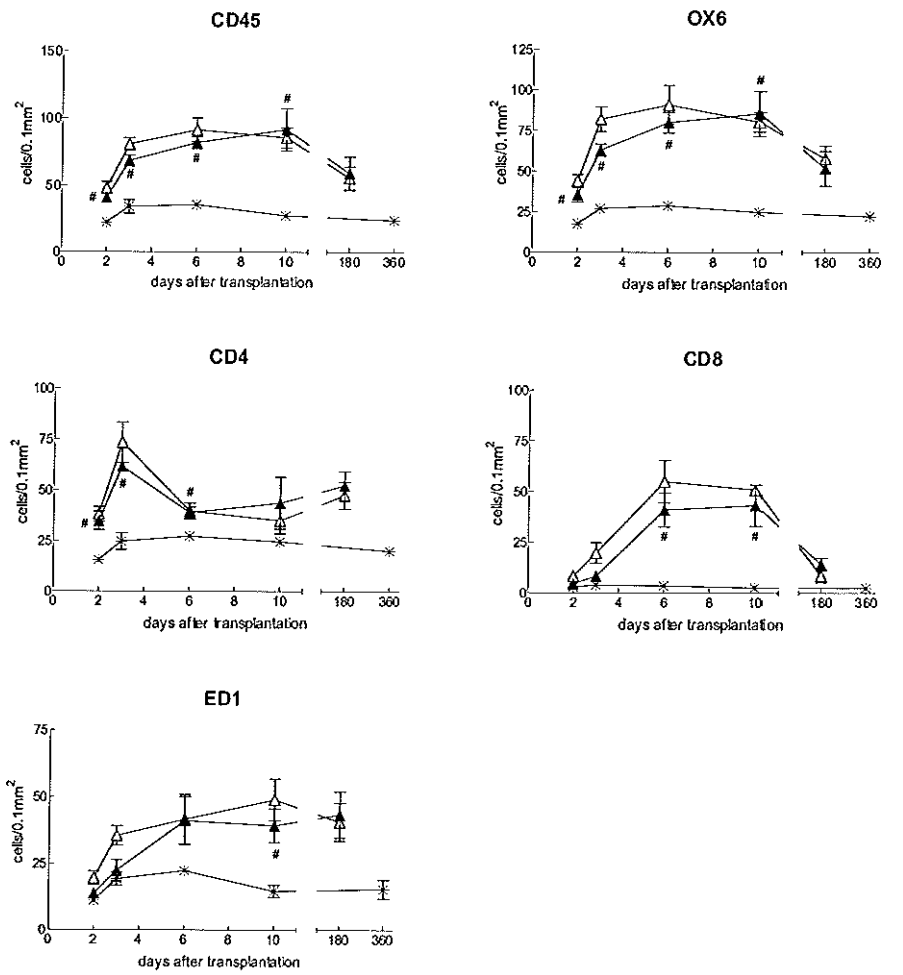

Figure 3B. Mononuclear cell infiltration under CsA therapy in non-ischemic allografts (▲) was abundantly present by day 2. At first $CD4^+$ cells invaded the graft, shortly followed by $CD8^+$ lymphocytes and ED-1^+macrophages. Hundred eighty days post-transplant, the allograft was mainly infiltrated by $CD4^+$ cells and ED-1^+ macrophages. Subjection of the renal allograft to 24h of cold ischemia (△) did not change the onset or severity of the cellular infiltration. CsA immunosuppressed, non-ischemic isografts (✲) served as controls. # = $p<0.05$ vs isograft,CsA

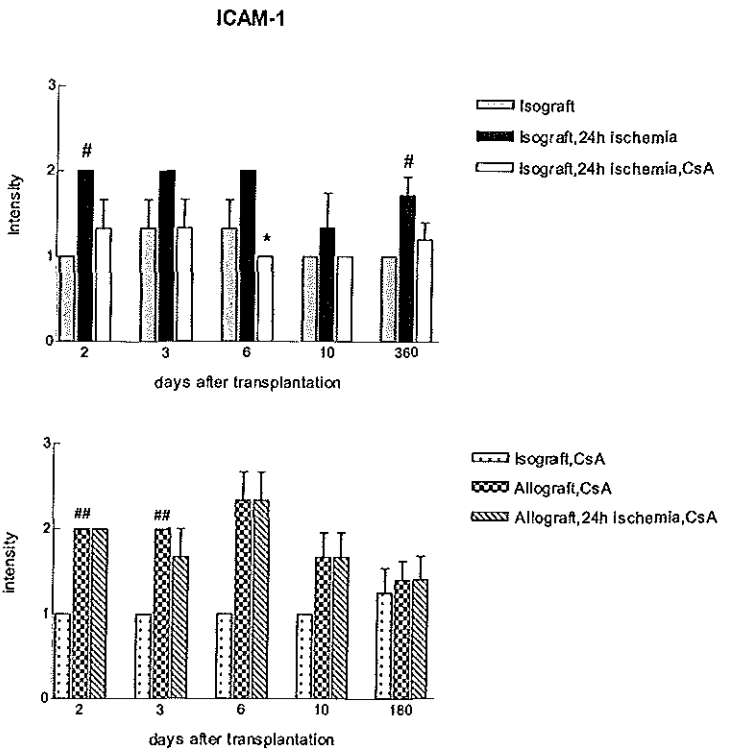

Figure 4. ICAM-1 expression in kidney iso-and allografts, subjected to 24h of cold ischemia. The intensity on the endothelium and tubules was measured semiquantatively on a 0-3 scale (0= none, 1= mild, 2= moderate, 3= dense). # = p<0.05 vs isograft; * = p<0.05 vs isograft,24h ischemia; ## =p<0.05 vs isograft,CsA

Cold ischemia in Allografts

Under CsA treatment, non-ischemic allografts demonstrated significantly higher expression of ICAM-1 (Figure 4) and more MHC class II$^+$ cells at day 2 compared to isografts (Figure 3B), and the number of these cells progressively increased until day 6. These MHC II$^+$ cells mainly consisted of CD4$^+$ cells and ED1$^+$ macrophages at day 2 and 3, and from day 3 on of CD8$^+$ cells and ED1$^+$ macrophages, while numbers of CD4$^+$ cells decreased. By week 26, the extent of MHC class II infiltration had diminished, predominantly due to a reduction of CD8$^+$ cells. CD4$^+$ cells and macrophages were localised primarily in the

glomeruli, peritubularly and around vessels. Also, some $CD8^+$ cells were seen in the glomeruli and perivascularly.

Twenty four hours ischemia of allografts did not affect the onset or progression of cell infiltration in the first 10 days. At week 26, there were still no significant differences in cell numbers between in both groups.

Discussion

In unravelling the etiology of CTD, some clinical studies on cadaveric donor kidneys demonstrated that prolonged cold ischemia shortened late graft survival.[57] Another indication that cold ischemic preservation injury is implicated in the process leading to CTD is the superior long-term survival of living donor transplants, both related and unrelated compared to that of cadaveric donor kidneys.[8] Since reliable comparisons between clinical studies are hindered by a variety of used preservation solutions, storage methods, immunosuppressive regimens as well as a different donor status, we studied the effect of prolonged cold ischemia in an experimental kidney transplantation model in the rat, both in a syngeneic and allogeneic setting.

Our study demonstrates that 24 hours of cold ischemia induces CTD in syngeneic kidney transplants. Three days after transplantation, endothelial cells were swollen and tubular damage was evident. Concurrently, the adhesion molecule ICAM-1 was upregulated on endothelial and tubular cells and coincided with an increase of MHC class II^+ infiltration, mainly consisting of $CD4^+$ T lymphocytes and macrophages. After a functional recovery, the ischemic isografts showed from week 18 onwards a progressive proteinuria. One year posttransplant, these kidneys demonstrated more glomerulopathy and had, in contrast with non-ischemic isografts, intimal hyperplasia. The degree of tubular atrophy and interstitial fibrosis was comparable. These observations concur with findings in a non-transplant rat model, showing that renal warm ischemia led to late functional and morphologic changes.[9] The significance of the infiltration of T lymphocytes and macrophages after ischemia/reperfusion in our study needs further investigation. Azuma *et al.* already demonstrated in the ischemic non-transplanted kidney that both cell types were activated, producing cytokines and growth factors like IL-1, TNF-α, PDGF, and TGF-β.[9] Several of these molecules may contribute to tissue injury, repair and the late fibrotic changes.

In order to understand the relevance of cold ischemic injury versus donor-recipient histoincompatibility, the BN donor kidney subjected to 24 hours of cold ischemia was transplanted into the WAG recipient, a fully MHC-mismatched combination. Non ischemic BN-WAG allografts immunosuppressed for 3 weeks with a relatively low dose of CsA developed in 26 weeks histopathological alterations that are compatible with CTD both in man[1] and rat.[2,17,18] Exposure of the donor kidney to 24 hr of cold ischemia in the allografts, however, did not affect the onset and progression of proteinuria and parenchymal lesions: The degree of glomerulopathy, tubular atrophy and interstitial fibrosis was comparable to that in non-ischemic allografts. In the first 10 days after engraftment, these ischemic kidneys showed similar, abundant numbers of lymphocytes and macrophages as non-ischemic controls. These observations conflict with data from Yilmaz *et al.* who showed in the DA-WF kidney allograft model that 60 minutes ischemia led to higher creatinine levels and more glomerular and vascular pathology than 30 min ischemia.[10] A likely explanation for this difference is that the cold ischemic insult was much greater after perfusion and storage with normal saline, as used in their study, than with UW-solution.

The apparent different outcome of ischemic injury on isografts and allografts appeared to be related to CsA treatment. Administering the same immunosuppressive regimen to ischemic isografts led to inhibition of cold ischemia induced late renal dysfunction. Long-term function and histomorphology were similar in both ischemic and non-ischemic CsA treated isografts. While CsA protects against ischemic induced early dysfunction in organs, as liver, small intestine, heart and brain,[19-22] the benefit of CsA in ischemic kidneys has not yet been reported. Some experimental studies showed that the early function and morphology of a warm ischemic kidney worsens after administration of CsA in a dose dependent way.[23,24] In our study, the relatively low dose of CsA did not necessitate to extend the 3 weeks period of support by the recipient's own kidney. In fact, CsA lowered the influx of $CD45^+$ leucocytes ($CD4^+$ lymphocytes and macrophages) in the post-ischemic kidney, a feature which is also observed by Ysebaert *et al.*[25] The mechanism of action of CsA in this non-alloimmune mediated inflammation is poorly known. A decrease of transendothelial migration of leucocytes[26] possibly by inhibition of adhesion molecule expression.[27-29] are reported effects of CsA. In our model, CsA also tended to inhibit the increased ICAM-1 expression after cold ischemia. Blockade of adhesion-molecules in the ischemic kidney is known to prevent the

inflammatory cascade and late dysfunction.[30] CsA could also act in ischemia-reperfusion injury both through direct action on T-cell infiltration and activation. Takada *et al* demonstrated that ischemia-induced late renal dysfunction was prevented by blockade of the T-cell CD28-B7 costimulation which inhibited T cell and macrophage infiltration and activation.[31] Thus, it seems that CsA inhibited the ischemic mediated response-to-injury in the allografts, but could not prevent the far greater alloimmune mediated response.

In conclusion, prolonged cold ischemia causes late kidney graft dysfunction, which is effectively inhibited by CsA. Under CsA treatment, prolonged cold ischemia does not appear to increase the immunogenicity of the allografts. The much earlier onset and progression of CTD in allografts than in isografts with prolonged cold ischemia reflects the major importance of the alloantigenic stimulus in the process.

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5

Cold ischemia augments allogeneic mediated injury in rat kidney allografts

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Summary

Background: We recently demonstrated that prolonged cold ischemia did not influence the onset or progress of chronic transplant dysfunction (CTD) in kidney allografts under Cyclosporine therapy, whereas extended ischemic times in untreated isografts led to CTD. To study whether Cyclosporine was responsible for this effect, the aim was to investigate the impact of ischemia on the immune response in rat kidney BN-WAG allografts and WAG-WAG isografts, both in the absence of immunosuppression.
Materials and Methods: To induce ischemic injury, donor kidneys were preserved for 24 hr in 4°C University of Wisconsin solution before transplantation. The histomorphology according to the BANFF criteria for acute rejection, and infiltrating cells ($CD4$- and CD8-cells, macrophages (MΦ)), MHC class II and adhesion molecule expression (P-selectin, ICAM-1) were assessed. Grafts were examined at day 1, 2, 3, 4, 6, and 8.
Results: At first cell infiltration of the interstitium accompanied by tubulitis was found, and later glomerulitis and vasculitis developed. In the allografts, exposure of the kidney to ischemia led to a significantly earlier onset of interstitial cell infiltration and tubulitis compared to non-ischemic allografts: BANFF score of interstitial cell infiltration was 1±0 vs 0.25±0.29 (day 3) and 2±0 vs 1.25±0.25 (day 4). Ischemic isografts showed oedematous endothelium and tubular damage from day 2 to 4, which was not observed in non-ischemic ones. From day 6, the histologic differences between ischemic and non-ischemic allo- and isografts disappeared. Ischemia led to more intense expression of P-selectin (day 1), ICAM-1 (day 2), and MHC class II on endothelium and proximal tubular cells (day 2). Concurrently with the upregulated ICAM-1 and MHC expression, significantly more $CD4^+$ cells and MΦ infiltrated the ischemic allografts at day 2 and 3. In isografts, exposure to prolonged ischemia also led to a higher influx of $CD4^+$ cells and MΦ than in non-ischemic isografts. Nonetheless, at day 2 and 3, the difference in number of MHC class II^+, and $CD4^+$ cells between ischemic and non-ischemic allografts was significantly greater than between both groups in the syngeneic setting.
Conclusion: Cold ischemia augments allogeneic mediated cell infiltration in rat kidney allografts. The earlier onset of acute rejection in these allografts may be prevented by better preservation, or by more adequate immunosuppressive therapies.

Introduction

Prolonged cold ischemia has been inconsistently identified as a risk factor for chronic transplant dysfunction (CTD) in human solid organ transplantation.[1-4] Using a syngeneic kidney transplantation model, we recently demonstrated that prolonged ischemia is an independent factor for the development of CTD.[5] It has also been suggested that ischemia predisposes to acute rejection, the latter being the most consistent risk factor for CTD.[6] Some clinical studies demonstrated that organ grafts subjected to prolonged cold ischemia or with delayed function, a parameter which correlates with the ischemic period[2,3,7,8] experience more often an early acute rejection episode than grafts which functioned immediately.[2,7,9,10] Some centres indicate that the effect of ischemia on CTD takes only place via this route: Troppmann *et al.* showed that delayed graft function in the absence of acute rejection did not affect long-term outcome.[11] Therefore, it has been hypothetised that ischemia initiates an inflammatory response that provokes increased host immunological reactivity, possibly by upregulated MHC expression on graft tissue after ischemia.[12] One recent experimental study showed that prolongation of the ischemic insult from 4 hours to 24 hours resulted in higher MHC class II antigen expression and more severe rejection of lung allografts in immunosuppressed dogs.[13] In contrast, in our kidney transplantation model, prolonged cold ischemia did neither affect adhesion-molecule and MHC antigen expression, and histomorphology during the first 10 days in Cyclosporine-treated allografts, nor did it influence the onset or progress of CTD.[14] It may be speculated that the absence of the deleterious effect of prolonged ischemia may be due to treatment with Cyclosporine.

To investigate this, the aim of present study was to study the impact of ischemia on the immune response to renal allografts and isografts in the absence of immunosuppression.

Materials and methods

Animals

Male rats of the inbred Brown Norway (BN)(RTI^{n}) and WAG/Rij (RTI^{u}) were purchased from Harlan, Austerlitz, The Netherlands. The rats weighed 200-250 g and 2-3 months of age at the beginning of the experiment. 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.[15] The animals were anaesthetised 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 of 4 °C with a rate of 2 ml /min. The kidney was excised and stored in UW solution 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. During surgery, the graft was wrapped in gauze moisturised with 4°C phosphate buffered saline (PBS). The perioperative ischemic time was 30 minutes. After revascularization, the ureter was anastomosed end-to-end to the distal third part of the recipient's ureter using single 11-0 prolene sutures. The ipsilateral native kidney was removed at time of transplant, whereas the contralateral one remained untouched until sacrifice. The mean survival time for kidney allografts without immunosupression in a bilaterally nephrectomized recipient in this strain combination is 10±2 days.

Cold ischemia period

The donor kidney was stored at 4°C for either 10 minutes, i.e. the minimal time in which the recipient is anaesthetised and prepared for implantation of the graft, or 24 hours. The 10 minutes cold ischemia will subsequently be referred to as non-ischemia.

Experimental groups

The impact of prolonged cold ischemia on the development of acute rejection was studied in BN-WAG donor-host combination without use of immunosuppression. Non-ischemic BN-WAG allografts, and ischemic and non-ischemic WAG-WAG isografts served as controls to determine the relationship between ischemia and allogenicity.
Representative specimens of kidney grafts were removed and snap frozen in

liquid nitrogen for immunohistology. Kidney specimens were also placed in 3.6% buffered formaldehyde for histomorphology.
Morphological and immunohistological evaluation was performed at day 1, 2, 3, 4, 6, and 8 post-transplantation (n=5 animals/group/timepoint for allografts, n=3 animals/group/timepoint for isografts).

Macroscopy

At the time of sacrifice, the ureter was inspected for its diameter. The kidney transplants were examined for signs of acute rejection: swelling, haemorrhage and infarction. Kidney grafts with early hydronephrosis or pyelonephritis were excluded from the study.

Histology

Paraffin-embedded 3 µm sections were cut and a haematoxylin/eosin, a periodic acid Schiff (PAS) and a Jones-staining was performed. Histological analysis was performed using the BANFF criteria for acute rejection[16] in a blind fashion.

Immunohistology

Representative portions of all kidneys were stained on 5 µm cryostat sections by a three-layer immunoperoxidase technique. After fixation with acetone for 10 minutes, tissues were dehydrated through graded alcohols to block endogenous peroxidase activity by incubation in methanol/0.03% H_2O_2 for 10 minutes. 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 one hour 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), Natural Killer cells (3.2.3), major histocompatibility complex (MHC) class II antigens (OX-6), and intercellular adhesion molecule 1 (ICAM-1). After each incubation, slides were washed in PBS-Tween 20, 0.1%. A second layer, rabbit anti-mouse IgG (Dako) was then applied for 30 minutes and after washing, slides were incubated with the third layer, mouse peroxidase-anti peroxidase (Dako) for 30 minutes. After washing in PBS, the reaction was developed by the addition of Diaminobenzidine substrate (Dako) for 8 minutes and slides were counterstained in Mayer's hematoxylin for 40 sec, washed, dehydrated and mounted. In order to stain the rabbit polyclonal antibody P-selectin (Pharmingen,

San Diego, CA) the preincubation was done with Normal Swine serum (Dako), and after staining the sections were incubated with swine-anti rabbit IgG (Dako) and subsequently with rabbit peroxidase-anti-peroxidase (Dako).

The immunohistochemical analysis was done in a blind fashion by two observers. Positive cells were counted in at least 8 fields with a 400X magnification and expressed as number of positive cells/0.1 mm^2. P-selectin and ICAM expression was analysed semiquantatively in different cortical regions: glomerulus, peritubular, perivascular, and intravascular. A scale was made ranging from 0 (none), 1 (very mild), 2 (mild), 3 (moderate), to 4 (dense).

Statistics

For statistical analysis of ordinal data (macroscopy, histopathology and adhesion-molecule expression) Kruskal-Wallis-one way ANOVA followed by Mann-Whitney test was performed. One way ANOVA followed by a NewMan Keuls test was used for the data of infiltrating cells. A 95% confidence interval for difference between means was calculated for cell infiltration in ischemic and non-ischemic kidneys both in the allogeneic and syngeneic groups. Then, the confidence interval of the allogeneic groups was compared to that of the syngeneic groups: When the maximum level of one group did not reach the minimum level of the other group, the intervals were defined to be significantly different. Statistical significance was established at $p<0.05$. The results are expressed as mean ± SD in the text, and as mean ± SEM in the figures.

Results

Macroscopical appearance

In both the ischemic and non-ischemic allografted groups, the ureters had a normal calibre, with no signs of obstruction or leakage throughout the 8 days follow-up. Until day 4, the ischemic kidney allografts had a normal appearance. Six days after transplantation, however, 5 out of 7 rats were found to have a swollen kidney, with diffuse haemorrhages in the parenchyma. The renal artery was open in all cases; the ureter had a normal calibre. In non-ischemic allografts only 1 out of 6 kidneys showed the same changes at day 6. ($p=0.06$) Two days later, no differences between both groups could be observed: All kidneys in both groups were rejected. The ureter and kidney from both ischemic and non-

ischemic isografts had a normal appearance at all timepoints studied.

Histology

The results analysed according to the BANFF criteria are depicted in Figure 1. The histologic course in the allografts was as follows: At first infiltration of the interstitium is observed, and thereafter tubulitis, glomerulitis and vasculitis are found. Exposure of the donor kidney to 24 hr of cold ischemia led to an earlier onset of the interstitial cell infiltration and tubulitis (Figures 1 and 2). At day 2, some ischemic allografts showed infiltrating cells and tubulitis, and at day 3 all of these grafts had grade 1 interstitial cell infiltration, which was significantly higher than that in non-ischemic allografts. Four days after engraftment, the degree of tubulitis was significantly higher in the ischemic allografts. From day 6, the interstitium of ischemic as well as non-ischemic allografts was completely

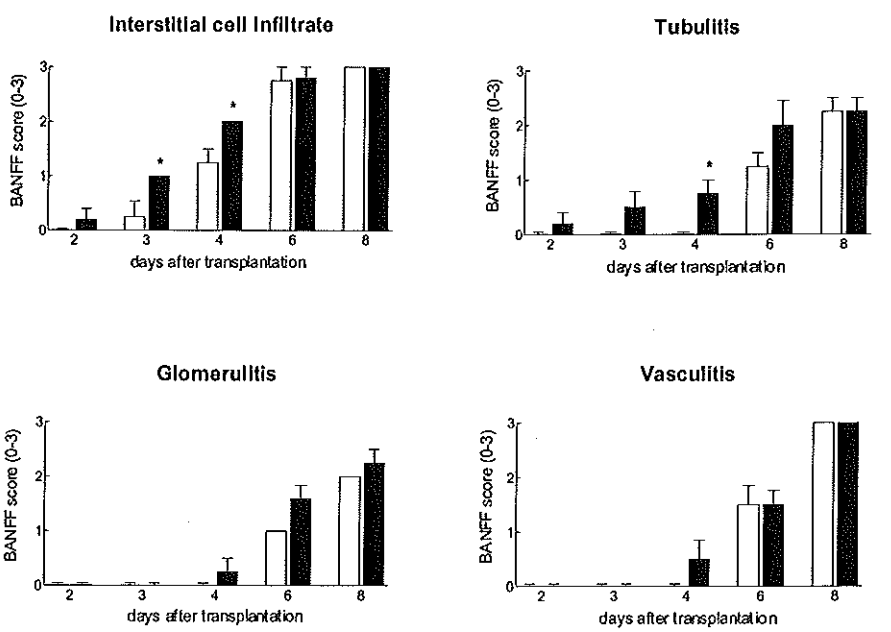

Figure 1. Severity of histopathological lesions in ischemic kidney allografts
Allografts preserved for 24 hr (■) demonstrated a higher degree of interstitial cell infiltration at day 3 and 4 after transplantation than non-ischemic allografts (□) (* = $p<0.05$). In addition, the onset of tubulitis was earlier, which attained a significance at day 4 (* = $p<0.05$). In isografts, ischemia led to minimal histologic changes: both in ischemic and non-ischemic isografts, the BANFF scores were 0.

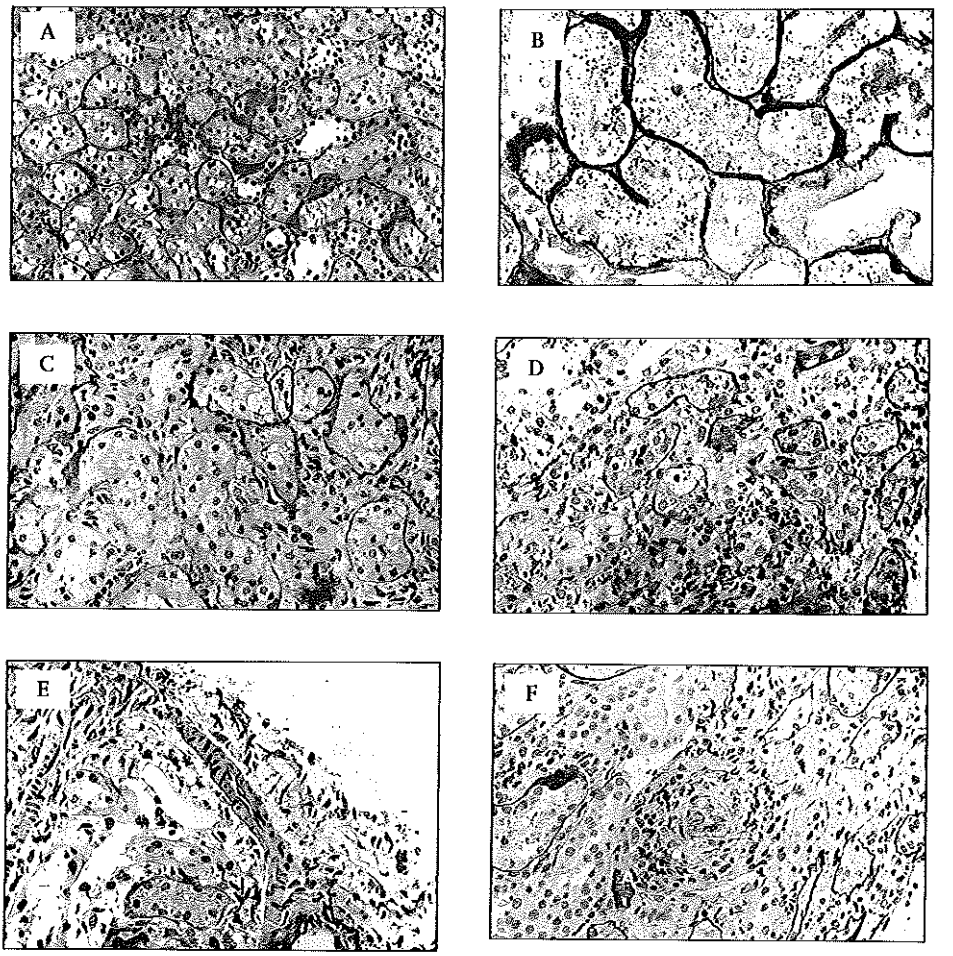

Figure 2. A) Normal tubulointerstitium of a kidney isografts. B) Lysosomal enzymes in tubulus cells of a 24 hr preserved kidney isograft at day 3. C) Mild tubulitis and cellular infiltration in a kidney allograft at day 3. D) Moderate to severe tubulitis and cellular infiltration in a 24 hr preserved kidney allograft at day 3. E)A normal intraparenchymal artery in a kidney allograft at day 4. F) Endothelial swelling and perivascular cell infiltration in a 24 hr preserved kidney allograft at day 4.

infiltrated with host mononuclear cells. By day 6, glomeruli and vessels were becoming involved in the process, leading to a full blown acute rejection at day 8 with haemorrhagic infarctions in at least 70% of the kidney, sometimes with thrombi in the midsize intraparenchymal arteries. Interestingly, 2 out of 7 ischemic allografts had beginning fibrosis in the interstitium, which was not seen in the non-ischemic group.

Non-ischemic isografts showed no histologic changes during this period, whereas ischemic isografts showed some endothelial and tubular damage at day 2 to 4. The endothelium was oedematous and some tubules had protein casts and lysosomal enzymes, observations that did not change the BANFF score in the ischemic isografts compared to the non-ischemic ones. From day 6 and onwards, no difference could be observed between ischemic and non-ischemic isografts.

Immunohistology

In the non-ischemic allografts, the adhesion molecule P-selectin increased progressively in the peritubular capillaries during the first 6 days. By day 6, the glomeruli and the endothelium of intraparenchymal arteries also expressed P-selectin. Exposure of the kidney to 24 hr of cold ischemia did increase P-selectin expression, although it did not reach significance the allogeneic setting. (Figure 2). In addition, in ischemic kidneys the expression of adhesion molecule ICAM-1 was upregulated by day 2 (Figure 2). Thereafter, in the allografts ICAM expression increased progressively, but the intensity between ischemic and non-ischemic grafts was comparable. From day 2 and onwards, ischemia also induced expression of MHC class II antigens on the peritubular endothelium, and increased its expression on the proximal tubular cells, in iso- and allografts to a similar degree. Concurrently with the upregulated ICAM-1 and MHC class II expression on graft tissue, significantly higher numbers of $CD45^+$ leucocytes infiltrated the ischemic allografts at day 2 and 3 (Figure 3). Many of these leucocytes were MHC class II^+ cells, $CD4^+$ cells and $ED1^+$ macrophages (Figure 3). Although the number of MHC class II^+ cells did not change after day 4, the ratio of T lymphocytes CD4 and CD8 subsets were quite different between ischemic and non-ischemic allografts. While the ischemic grafts had a maximal infiltration of $CD4^+$ cells at day 3, with a rapid decline thereafter, the non-ischemic allografts showed the highest number at day 4. Whilst the number of $CD4^+$ cells declined, the number of infiltrating $CD8^+$ T lymphocytes progressively increased. $ED1^+$ macrophages infiltrated the ischemic graft in

higher numbers, but after day 3 the difference in number between ischemic and non-ischemic allografts disappeared. NK-cells infiltrated the grafts at the late stage of acute rejection. By day 6 some cells were present, both in ischemic and non-ischemic kidneys, 23±17 and 15±5 NK cells/0.1mm², respectively (p=0.42).

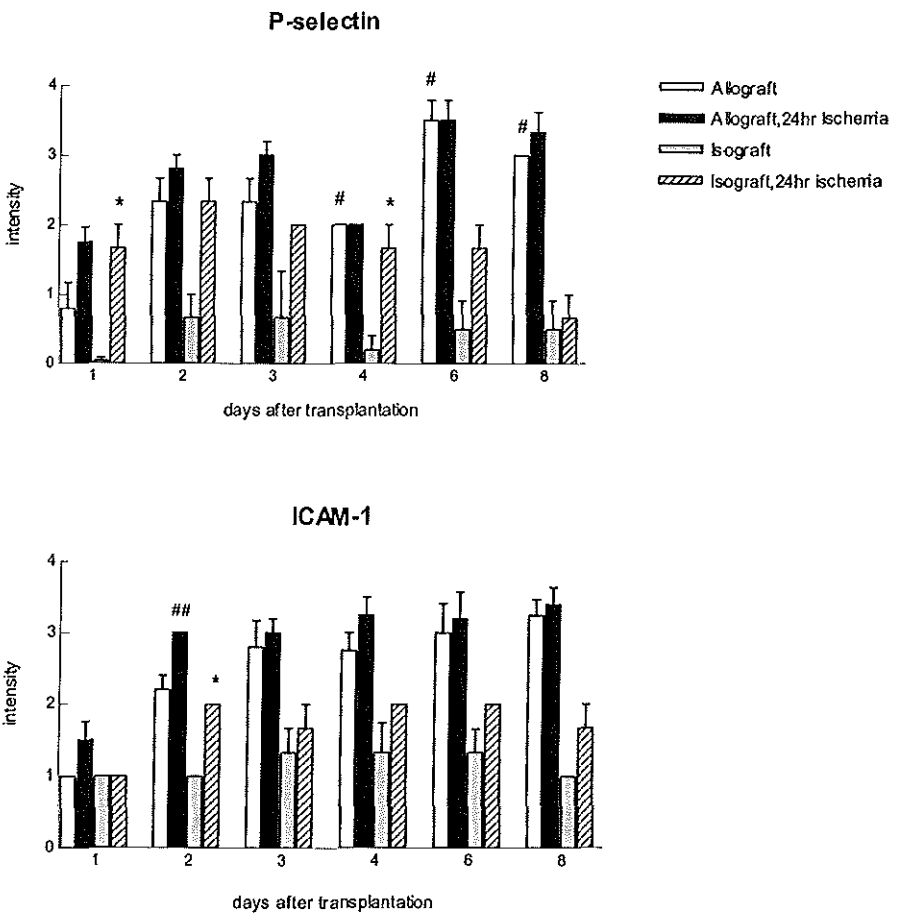

Figure 2. P-selectin and ICAM-1 expression in ischemic kidney iso- and allografts.
The immunohistochemical intensity of the adhesion molecule expression was measured semiquantatively on a 0-4 scale. (0=none, 1=very mild, 2=mild, 3=moderate, 4=dense). At day 1, P-selectin was increased after ischemia in isografts (* = p<0.05). In allografts, the expression of P-selectin was higher than in isografts (# = p<0.05). Ischemia did not affect P-selectin expression in allografts. ICAM-1 expression increased at day 2 after ischemia (* = p<0.05) in isografts. (## p<0.05 vs isograft,24hr ischemia)

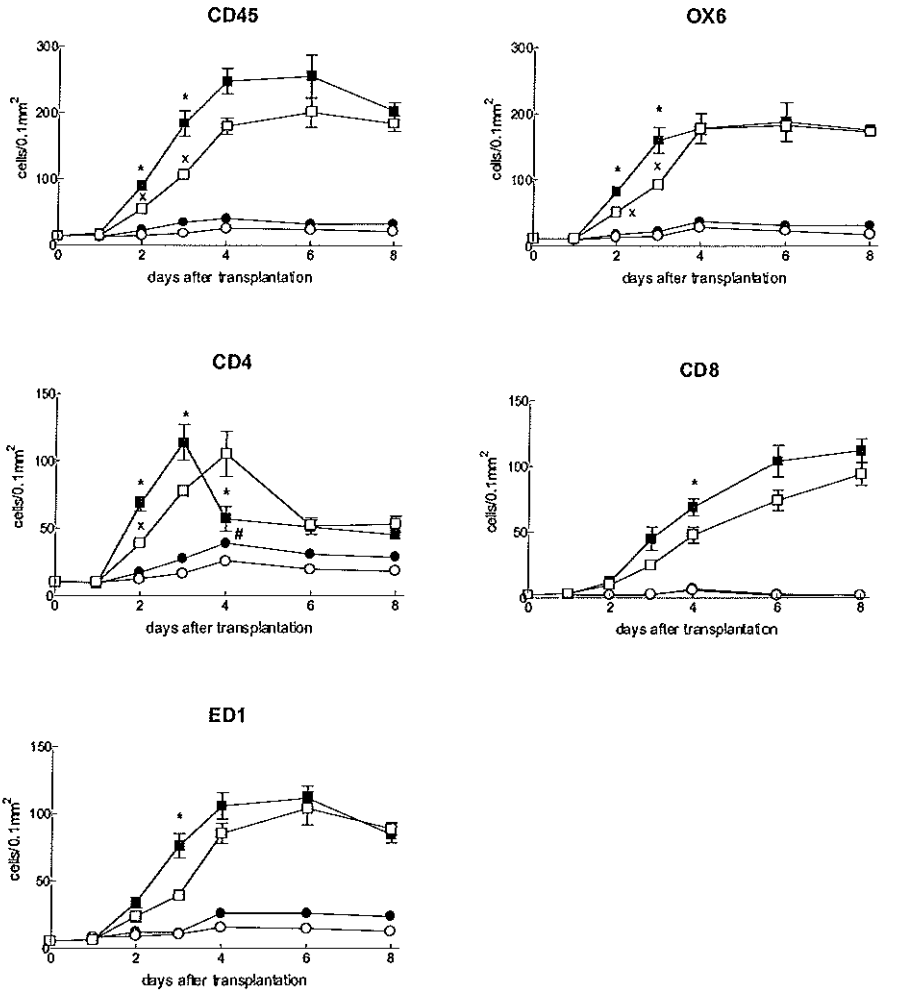

Figure 3. Cell infiltration in ischemic kidney iso- and allografts.
In allografts, subjection of the kidney to 24 hr of cold ischemia (■) led to an increase of cell infiltration compared to non-ischemic controls (□).In the first 3 days, $CD45^+$ leucocytes consisted mainly of MHC class II^+, $CD4^+$ cells and ED-1^+macrophages. The peak of $CD4^+$ cells was one day earlier, and the number of cytotoxic $CD8^+$ T cells was higher at day 4. By day 6, the difference in cell infiltration between both groups disappeared. Cell infiltration into ischemic isografts (●) is also increased from day 3 and onwards compared to non-ischemic isografts (○), except for $CD8^+$ T-cells (# = $p<0.05$). The increase of infiltration of MHC class II^+ and $CD4^+$ cells after ischemia was significantly higher in allografts than in isografts at day 2 and 3 post-transplantation, when compared to their non-ischemic counterparts. (X = in 95% the maximum confidence interval between the means of ischemic and non-ischemic isografts do not reach the minimum confidence interval between the means of ischemic and non-ischemic allografts.)

In isografts, exposure to prolonged ischemia led also to a higher influx of $CD45^+$ leucocytes, mostly consisting of $CD4^+$ cells and $ED1^+$ macrophages than in non-ischemic isografts. Nonetheless, at day 2 and 3, the difference in number of $CD45^+$, MHC class II^+, and $CD4^+$ cells between ischemic and non-ischemic allografts was significantly greater than between both groups in the syngeneic setting.

Discussion

In the present study, we demonstrate that prolonged cold ischemia results in an earlier onset of the histological and immunological features of rejection in kidney allografts. The mechanism through which ischemia increases the allogeneic immune response is not fully understood. Whilst in isografts, 24 hour of ischemia led to an increase of the early adhesion molecule P-selectin at day 1 and ICAM-1 at day 2 on peritubular capillaries, in allografts ischemia did not further affect their expression: expression of these adhesion-molecules was persistently increased in both ischemic and non-ischemic allografts. Increased P-selectin and ICAM-1 expression could be due to proinflammatory cytokines released by ischemia activated endothelium. This may mediate the selective migration of cell populations across endothelial barriers of the graft. Cytokines (IL-1, IFN-γ) of recruited and activated $CD4^+$ cells, which numbers dramatically increased after day 1 in allografts, could be responsible for the ongoing endothelial cell expression of adhesion molecules in allografts.

In our study both ischemic allo- and isografts had an induced expression of MHC class II on vascular endothelium and increased expression of MHC class II on the proximal tubular cells from day 2 and onwards. Shoskes *et al.* also demonstrated that transient ischemia in a non-transplant kidney led to an early upregulation of MHC class I and II mRNA transcripts and protein expression on tubular cells.[12] This observed MHC class II hyperexpression - triggered by release of IFN-γ and other mediators [17,18] -, led to the hypothesis that ischemia could increase the immunogenicity of a organ graft. Recently, this suggestion has been fortified in an experimental lung allograft model: Lung allografts with prolonged cold ischemia had an increased expression of MHC class II antigens on the vascular endothelium and bronchial epithelium and a more severe rejection compared to allografts with a short ischemic period.[13] Since ischemic lung

isografts were not included in this study, the higher degree of histological rejection in ischemic lung allografts cannot be exclusively attributed to increased allo-immunogenicity by ischemia: Ischemia induced histologic damage could be superimposed on allogeneic mediated damage. Our data indicate that ischemia augments the alloimmune response, since the increase of infiltration of MHC class II$^+$ and CD4$^+$ cells was significantly higher in allografts then in isografts at day 2 and 3 post-transplantation, when compared to their non-ischemic counterparts. In addition, in ischemic allografts interstitial cellular infiltrates and tubulitis developed earlier in the process of histological rejection, whereas ischemia in isografts led to only minimal histologic changes: oedematous swelling and tubules with protein casts and lysosomal enzymes.

Interestingly, the increased CD4$^+$ T cell infiltration at day 2 and 3 in allografts did not correlate with the degree of MHC class II antigen expression on graft tissue, since allografts had a similar expression as isografts. Moreover, in contrast to the MHC class II antigen expression on endothelial cells that together with costimulatory molecules activate CD4$^+$ T cells,[19] the relevance of MHC class II hyperexpression on tubular epithelium is less clear. Epithelial cells do not seem to activate CD4$^+$ cells,[20,21] because they miss costimulator molecules, such as B7. Nevertheless, even in a non-allogeneic setting, ischemia appears to activate CD4$^+$ T cells: It has been found that blocking the CD28-B7 costimulatory pathway of T cells inhibits early and late ischemic kidney injury effectively.[22]

In conclusion, ischemia augments allogeneic mediated cell infiltration, which relevance in terms of cytokine expression is currently under investigation. The earlier onset of acute rejection in these allografts may be prevented by better preservation, or by more adequate immunosuppressive therapies.

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6

Uretero-neocystostomy contributes to late functional and morphologic changes of rat kidney transplants

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Submitted for publication

Summary

Purpose: To investigate whether the type of surgical techniques of ureter reconstruction could have an impact on late function in kidney transplants, the uretero-neocystostomy (U-B) and uretero-ureterostomy (U-U) were compared. To rule out alloantigeneic mediated effects on late graft dysfunction, we transplanted kidneys syngeneically.
Materials and methods: Rat kidney isografts were transplanted with either a U-B or a U-U. Unilaterally nephrectomised (UNx) rats served as controls. At 8 weeks post-transplantation, the intrapelvic pressure was measured under basal diuresis, after intravesical, and after intrapelvic infusion. Albuminuria was determined monthly until sacrifice at week 52. Histomorphological analysis included the degree of glomerulopathy, tubular atrophy, interstitial fibrosis and intimal hyperplasia. $CD4^+$-, $CD8^+$T cells and macrophages (MΦ)) were identified using immunohistochemistry.
Results: At 8 weeks, the intrapelvic pressure under basal diuresis and after intrapelvic infusion was significantly increased in U-B isografts compared to U-U isografts and UNx rats, whereas intravesical infusion did not change the pressure in any of the groups. During follow-up Ualb in U-U did not differ from UNx. In contrast, Ualb significantly increased in U-B isografts from week 36 onwards. At week 52, the ureter and kidney of U-U transplants and UNx had a normal appearance, whereas all ureters in the U-B group were dilated. Nevertheless, 6 out of 8 U-B kidneys had a normal appearance. However, the histomorphology of U-B transplants demonstrated significantly more interstitial fibrosis and more $CD\text{-}8^+$ T-cells and m compared to U-U isografts.
Conclusions: The U-B surgical technique for restoring the urinary tract after kidney transplantation contributes to the development of long-term functional and histological renal changes. A partial obstruction may be the cause of this renal impairment.

Introduction

Despite progressive improvement in 1 year graft survival rates - mainly due to progress in treating acute rejection episodes-, beyond 1 year the annual rate of kidney graft loss has not changed over the last 3 decades.[1,2] Late failure of kidney allografts is largely attributable to chronic transplant dysfunction (CTD), which histomorphology consists of glomerulopathy, tubular atrophy and interstitial fibrosis.[3] The pathogmonic characteristic is intimal hyperplasia, which can frequently not be assessed in biopsies because of absence of arteries.[4] The histomorphology of other causes of late dysfunction, like obstuctive nephropathy, can easily overlap with the non-specific kidney changes seen in CTD. Some clinical transplant studies demonstrate that urologic complications may have long-term effects on graft function.[5]

The continuity of the urinary tract in clinical renal transplantation is usually restored by uretero-neocystostomy. Notwithstanding the surgical technique of uretero-neocystostomy applied, the incidence of urologic complications has been reported to range from 1-10%.[6,7] The majority of these complications occur within the first month after transplantation. Late complications are predominantly ureteral obstruction and vesicoureteral reflux.[8]

A severely dilated pyelum is known to impair renal function and morphology, finally resulting in end stage renal disease.[9] Some clinical transplant studies have shown that mild dilation of the pelvicalyceal system occurs frequently, although it does not affect allograft function significantly.[10,11] However, negative effects of the surgically restored urinary tract on renal function and morphology in clinical transplants could be masked by overwhelming allogeneically mediated damage. Therefore, to study the impact of such a factor in a isolated fashion, an animal model in which can be transplanted in the absence of an allogeneic response, is necessary.

To investigate whether the type of ureter anastomosis could has an impact on late functional and morphological changes in the kidney transplants, we performed two different techniques of ureter reconstruction. The clinical most widely used uretero-neocystostomy and the uretero-ureterostomy.

Materials and methods

Animals

Male rats of the inbred Brown Norway strain (BN; RTI[n]) were purchased from Harlan, Austerlitz, The Netherlands. The rats weighed 200-250g and were 2-3 months of age at the beginning of the experiment. 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.

Transplantation technique

Kidney transplantation was performed from BN to BN using the technique described by Fisher and Lee.[12] The animals were anaesthetised with ether. After an intravenous injection of heparin (100 IU), the donor kidney was flushed *in situ* via the aorta with 5 ml University of Wisconsin (UW) solution of 4 °C at a rate of 2 ml /min. The kidney was excised and stored in UW solution (4°C) for about 6 minutes prior to implantation. 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. The total perioperative ischemia time was 30 minutes. Two methods of restoring the continuity of the urinary tract were compared.

Experimental groups

Group 1

BN-BN-kidney transplants with uretero-neocystostomy (U-B; n = 13). The U-B was performed as described by Fisher and Lee.[12] In brief, the donor ureter was directly inserted into the bladder and fixed with one 8.0 stitch to the dorsal vesical wall about 3 mm from the incision made at the apical site of the bladder.

Group 2

BN-BN-kidney transplants with uretero-ureterostomy (U-U; n = 10). The U-U was performed as described by Oesterwitz *et al.*[13] In brief, the ureter of the donor was anastomosed end-to-end to the distal third part of the recipient's ureter using 4 interrupted 10-0 prolene sutures, without the use of a stent.

In both groups, the left native kidney was excised at the time of transplantation.

Nephrectomy of the contralateral kidney was performed 3 weeks after transplantation.

Group 3
Unilaterally nephrectomised (UNx) BN rats (n = 13) served as controls.

The follow-up period was 8 weeks for intrapelvic pressure measurements (n = 5 for each group) and 52 weeks for functional and morphological studies of the kidney.

Intrapelvic pressure studies

Intrapelvic pressure was determined in all 3 groups at 8 weeks post-transplantation. Under ether anaesthesia, a midline abdominal incision was made. A 20 gauge catheter was introduced into the upper pole of the kidney graft and then pushed up towards the renal pelvis. The catheter was initially advanced into the upper ureter with visualisation of the catheter tip, then relocated in the renal pelvis for continuous pressure monitoring by a Uniflow pressure transducer (Baxter, United Kingdom). The zero-calibration of the pelvic pressure was done at the level where the catheter inserted the pyelum. Then, a 20 gauge catheter was placed into the ventral side of the bladder for bladder emptying. Renal pelvis and bladder were examined for leakage after placement of the catheters.

Vesico-ureteral reflux was tested by examining the ureter for retrograde flow of Evans blue: after emptying the bladder, it was filled with 0.7 ml phosphate buffered saline (PBS) with Evans blue, the maximum volume before spontaneous voiding occurred. The intrapelvic pressure was recorded concomitantly.

To detect ureter obstruction, the bladder was emptied and the intrapelvic pressure was measured. Subsequently, the pelvis was filled with 0.1 ml PBS with Evans blue via the renal catheter and the intrapelvic pressure was measured continuously for 30 min.

Renal function

Urine was collected monthly by placing the rats individually in metabolic cages for 24 hours. Protein excretion was colorimetrically by addition of pyrogallol red. Serum creatinine was determined using the Jaffé method without deproteinization.

Macroscopy

The kidney transplants were examined for hydronephrosis and pyelonephritis. The ureter was inspected for its diameter, its vascularisation and for fibrotic thickening.

Histology

At 52 weeks post-transplantation, kidneys were harvested, fixed by immersion for 48h in a 3.6% buffered formaldehyde solution after longitudinal bisection, and embedded in paraffin for histopathological studies. Sections (3 µm) were stained with hematoxylin-eosin (H&E), periodic acid Schiff (PAS) and were evaluated for glomerulopathy, tubular atrophy, interstitial fibrosis, and intimal thickening using the BANFF-score formulated for CTD.[14] Briefly, these 4 criteria were separately determined with a score ranging from 0 = normal, 1 = to 25% affected, 2= moderate, affecting 25-50%, and 3= more than 50% changes.

Immunohistology

Representative portions of all kidneys were stained on 5 µm cryostat sections by a three-layer immunoperoxidase technique. After fixation with acetone for 10 minutes, endogenous peroxidase activity was blocked by incubation for 10 minutes in methanol/0.03% H_2O_2, after dehydration through graded alcohols. 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 one hour incubation with the primary monoclonal antibodies W3/25, OX-8, and ED-1 (Serotec, Oxford, United Kingdom) for identification of $CD4^+$-, $CD8^+$ T-cells and macrophages, respectively. After each incubation, slides were washed in PBS-Tween 20, 0.1%. A second layer, rabbit anti-mouse IgG (Dako) was then applied for 30 minutes and after washing, slides were incubated with the third layer, mouse peroxidase-anti peroxidase (Dako) for 30 minutes. After washing in PBS, the reaction was developed by the addition of Diaminobenzidine substrate (Dako) and slides were counterstained in Mayer's hematoxylin for 40 sec, washed, dehydrated and mounted. The analysis was done in a blind fashion by two observers. Positive cells were counted and expressed as number of positive cells /0.1 mm^2.

Statistics

Statistical analysis was performed using Kruskal-Wallis one way ANOVA followed by Mann-Whitney test, $p < 0.05$ 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

Intrapelvic pressure

under basal diuresis

At 8 weeks post-transplant U-B isografts had significantly higher intrapelvic pressures than U-U isografts and UNx rats (Table 1).

	BN-UNx	BN-BN, uretero-ureterostomy	BN-BN, uretero-neocystostomy
Intrapelvic pressure (cm H_2O)	1.4 ± 0.4	2.1 ± 0.9	8 ± 4.7 *

Table 1. Intrapelvic pressure at basal diuresis *=p<0.05 vs BN-BN, uretero-ureterostomy and BN-UNx.

after intravesical infusion

In isografts with either U-B or U-U and UNx, injecting 0.7 ml Evans blue into the bladder did neither lead to any dye in the ureter nor to an increase of the intrapelvic pressure.

after intrapelvic infusion

Infusion of 0.1 ml PBS intrapelvically led to a significant rise of the intrapelvic pressure in all experimental groups. The pressure in the isografts with U-B was significantly higher than in the other 2 groups. Moreover, the pressure in the U-B transplants returned to normal basal diuresis value after 30 minutes, whereas in the U-U kidney transplants and in the UNx group it normalised at 10 minutes after infusion (Figure 1).

Renal function

The excretion of protein of kidney isografts with U-U did not differ from UNx during the 1 year follow-up (Figure 2). Also, serum creatinine did not change in

U-U compared to UNx (Figure 3). In contrast, isografts with U-B had significantly increased proteinuria from 36 weeks after transplantation and beyond and serum creatinine levels from week 44 and onwards compared to the isografts with U-U and UNx. (Figures 2 and 3).

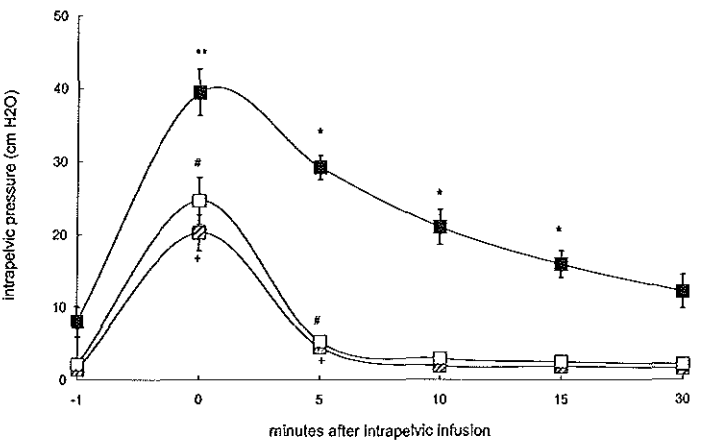

Figure 1. In syngeneic kidney transplants with either a uretero-ureterostomy (□) or a uretero-neocystostomy (■)and unilaterally nephrectomized BN rats (▨), the intrapelvic pressure was monitored after infusion of 0.1 ml PBS intrapelvically as described in the Materials and Methods. * / ** = p<0.05 vs the intrapelvic pressure of the uretero-neocystostomy at basal diuresis (-1 minutes). ** = p<0.02 vs the intrapelvic pressure of the uretero-ureterostomy-group and the UNx-group immediately after infusion (0 minutes). # = p<0.02 vs the intrapelvic pressure of the uretero-ureterostomy at basal diuresis (-1 minutes). + = p<0.02 vs the intrapelvic pressure of the Unx at basal diuresis (-1 minutes)

Macroscopy

At sacrifice, the ureter in kidney transplants with U-U had a normal calibre and vascularization.. At the side of anastomosis no fibrosis or stenosis could be detected. Furthermore, they did not demonstrate any sign of hydronephrosis or pyelonephritis. In contrast, all renal isografts with U-B had a dilated ureter, the ureteric wall was thickened and poorly vascularized. Nevertheless, only 2 out of the 8 kidney transplants showed signs of hydronephrosis. The other kidney transplants had a normal appearance. The ureters and kidneys in the UNx rats did not show any abnormalities.

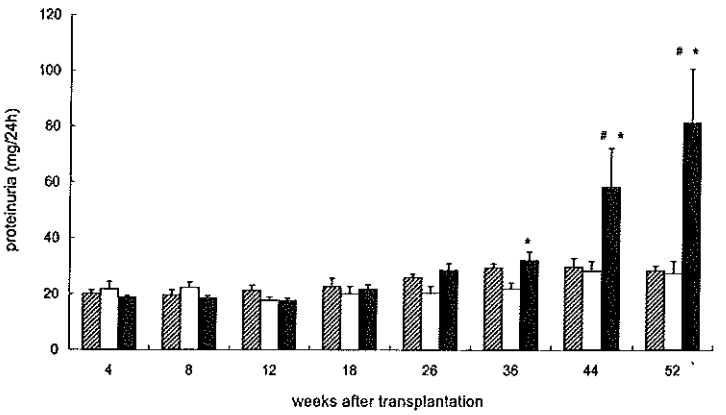

Figure 2. The excretion of urinary protein in kidney isografts with 2 types of urinary tract reconstruction: uretero-neocystostomy (■) and uretero-ureterostomy (□), and in unilaterally nephrectomized rats (▨).
* = $p<0.05$ vs isografts with uretero-ureterostomy and UNx at the same timepoint.

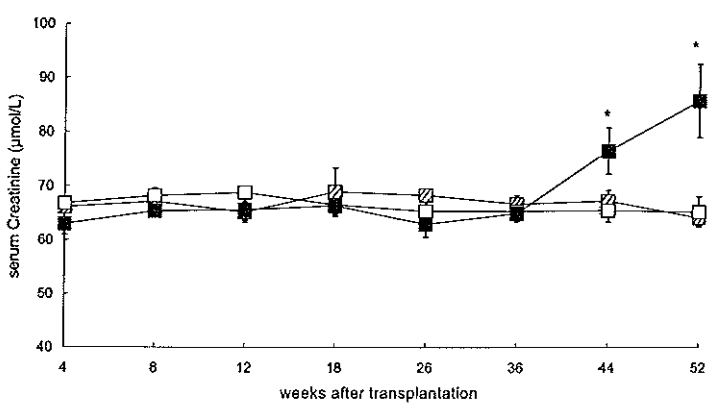

Figure 3. Serum creatinine concentrations in kidney isografts with 2 types of urinary tract reconstruction: uretero-neocystostomy (■) and uretero-ureterostomy (□), and in unilaterally nephrectomized rats (▨). * = $p<0.05$ vs isografts with uretero-ureterostomy and UNx at the same timepoint.

Histology

Isografted kidneys with U-U demonstrated some damage at week 52, which did not differ from that in BN-UNx; Banff-score was 2.6 ± 0.5 and 2.7 ± 0.8 respectively. As depicted in Figure 4, the lesions consisted of some glomerulopathy and tubular atrophy. Interstitial fibrosis and intimal thickening were not seen in any of the kidneys. In contrast, isografts with U-B had a mean BANFF score of 4.3 ± 1.0 which is significantly higher than that of the other 2 groups: The most striking difference was the presence of interstitial fibrosis in the U-B transplants (Figure 4). As in the kidneys of isografts with U-U and UNx, intimal hyperplasia was not seen, except minimal lesions in one kidney transplant.

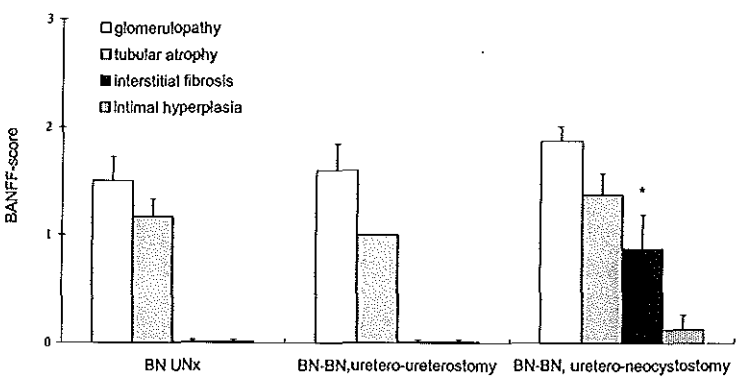

Figure 4. Histopathology of kidney isografts with 2 types of urinary tract reconstruction: uretero-neocystostomy and uretero-ureterostomy, and of unilaterally nephrectomized rats using the BANFF-criteria for chronic transplant dysfunction. * = $p<0.05$ vs isografts with uretero-ureterostomy and UNx.

Immunohistology

UNx-kidneys showed a few infiltrating $CD4^+$ and $CD8^+$ T-cells and ED-1 macrophages (MΦ), mainly localised peritubularly. Renal transplants with U-U had a similar distribution and quantity of the T cell-subsets and m. In isografts with U-B, there was a significant increase of $CD4^+$ and 8^+ cells and m in the interstitium (CD4: 44±12 vs 20±3 cells/0.1mm²; CD8: 15±6 vs 4±1cells/0.1mm²; m: 25±8 vs 12±3 cells/0.1mm²).

Discussion

In unravelling the etiology of late kidney graft failure, some clinical studies indicate that urologic complications may have an impact on late graft function and morphology. Until now little attention has been directed to the long-term effects of the surgical technique of ureteric anastomosis. Therefore, we studied the possible impact of 2 types of urinary tract reconstruction, the clinically most widely used uretero-neocystostomy (U-B) and the uretero-ureterostomy (U-U) on late kidney function and morphology.

The main finding of the present study is that the U-B induces late graft dysfunction in rat renal isografts, whereas the U-U does not. After an initial normal function, transplants with U-B demonstrated from week 36 onwards a progressive decline of renal function and significantly more interstitial fibrosis at week 52. Although it is generally known that urinary tract dysformities can lead to nephropathy, the U-B as the standard technique for reconstruction appeared to be reliable. The few clinical transplant studies on this subject could not demonstrate long-term adverse effects of the U-B per se on graft function. Even in dilated urinary collecting systems without obvious obstruction by antegrade pyelography, the serum creatinine did not differ from that in non-dilated renal allografts.[11] On the other hand, obstructed kidney allografts which were reconstructed secondary had serum creatinine levels at 3 months and 1 year which were significantly higher than in non-obstructed allografts.[11]

Although 6 out of 8 transplants with U-B had a normal appearing kidney and only 2 showed macroscopical signs of obstruction, the intrapelvic pressure under basal diuresis and after antegrade intrapelvic infusion was significantly increased. Therefore, a partial ureteric obstruction of the U-B isografts appeared to be the cause of renal impairment. This simultaneous occurrence of elevated intrapelvic pressure and normal pyelocalyceal system has also been observed in patients, in which it was indicated as cause of nephropathy.[15,16]

In contrast to acute ureteral obstruction with basal intrapelvic pressures of >20 cm H_2O causing immediate renal impairment,[17] in our study the increased intrapelvic pressure (8 cm) did not deteriorate renal function immediately. This was also found by others in an experimental model of partial ureteric obstruction, in which the glomerular filtration rate was not affected after 3 weeks, but was reduced by 60% after 1 year.[18]

The kidney isografts with U-B showed some glomerulopathy and tubular

atrophy, and significantly more interstitial fibrosis and interstitial infiltration of $CD4^+$ and $CD8^+$ T-cells and macrophages. These histologic and inflammatory features resemble the chronic tubulointerstitial nephritis in non-transplanted kidneys exposed to chronic urinary obstruction.[19]

With the present results, we show that the uretero-neocystostomy as method of restoring the urinary tract after kidney transplantation contributes to the development of long-term functional and histological renal changes. Although we used the BANFF criteria for CTD to analyse the morphology, the absence of intimal hyperplasia in the present study presumes a different pathophysiological origin. On the other hand, the aspecific changes - tubular atrophy and interstitial fibrosis - seen after uretero-neocystostomy could contribute to the overall histopathology in biopsy-proven CTD, thereby in fact overscoring the severity of CTD in allografts. Moreover, this study demonstrates that a non-dilated urine collecting system in kidney transplants does not guarantee appropriate urine outflow and that a partial obstruction can lead to late deterioration of kidney graft function.

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7

Genetic susceptibility of the donor kidney contributes to the development of chronic transplant dysfunction

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Summary

Introduction: Chronic transplant dysfunction (CTD) is the major cause of late organ graft loss. Since some transplant studies suggest differences in kidney graft outcome due to ethnicity, intrinsic genetic factors of the kidney may contribute to CTD.
Purpose: Therefore, the aim of the present study was to investigate possible differences in genetic susceptibility for CTD in a rat model. By transplanting different donor kidneys into a normotensive, histocompatible recipient, the kidneys were exposed to the same blood pressure profiles, metabolic and hormonal environment.
Materials and methods:Kidneys from young adult hypertensive fawn-hooded (FHH), a strain showing early onset chronic renal failure, normotensive August x Copenhagen-Irish (ACI) and (ACI x FHH)F_1 donors were transplanted into male F_1 recipients. The native kidneys of the recipients were removed 1 week after transplantation. The results were mutually compared and to their unilaterally nephrectomised littermates. Systolic blood pressure (SBP) and albuminuria (UaV) were determined at the time of transplantation and at 8 and 16 weeks. The histomorphological analysis included the incidence of focal glomerulosclerosis (FGS), and determination of CTD according to the BANFF criteria.
Results: A negative impact of the transplantation technique in this syngeneic situation could not be detected as F_1 transplants did not differ functionally and morphologically from their UNx controls. Transplanting an ACI kidney did not result in significant changes of SBP, UaV and incidence of FGS compared to F_1 transplants and ACI-UNx. In contrast, FHH kidneys did show a progressive increase of UaV and glomerulosclerosis and a significant higher BANFF score, whereas the SBP did not differ from F_1 transplants. The moderate hypertension seen in FHH did not travel with the kidney. Compared to the FHH-UNx, transplantation of a FHH kidney did significantly attenuate the rise of UaV and FGS.
Conclusions: The susceptibility of the donor kidney appears to be an important factor for the development of CTD.

Introduction

Chronic kidney transplant dysfunction (CTD) is the major cause of graft loss one year post-transplantation.[1,2] The etiology and pathophysiology of CTD are poorly understood. Generally, alloantigen-dependent factors as acute rejection are thought to be the main risk factors for CTD. However, alloantigen-independent factors also appear to contribute to functional and histopathological changes. Several donor- and recipient-related factors, such as age, renal mass, cold ischemia and hypertension have been implicated,[3,4] but an intrinsic genetic susceptibility of the donor kidney to renal damage may also be involved. End stage renal failure (ESRF) is a condition that shows great similarity to CTD both functionally and histopathologically. Several clinical studies analyzing the causes of ESRF suggest that genetic factors may play an important role in the risk of a hypertensive patient to develop ESRF. Hypertensive African-Americans, for example, have an 8- to 25-fold higher risk of developing ESRF than Caucasians, which cannot be explained by the severity or the treatment of hypertension.[5,6,7] Even more indicative for intrinsic genetic factors of the kidney are the findings of some transplant studies, which suggest differences in graft outcome due to donor ethnicity: Kidneys from African-American and Asian-American donors had significantly worse outcome than those from Caucasians.[8,9]

In an animal model, Churchill *et al.* clearly demonstrated that susceptibility of the kidney to hypertension-induced damage is genetically based. By exposing a transplanted Brown Norway (BN) kidney and one native kidney in a BN compatible-spontaneously hypertensive rat (SHR) to the same high blood pressure and metabolic environment, BN kidneys were found to be more susceptible to develop damage than SHR kidneys.[10]

Apart from a genetic susceptibility of the kidney to hypertension-induced damage, experimental transplantation studies also demonstrate that even in a normotensive environment the susceptibility of the graft to develop CTD appeared to be strain-dependent. Tullius & Tilney demonstrated significant proteinuria at week 52 in syngeneic Fisher rat transplants compared to unilateral nephrectomized Fisher rats,[11] whereas we did not observe any increase using BN isografts.[12]

Genetic factors influencing the development and progression of renal damage have been suggested, but specific genes have not emerged. Recently, we have found genes in the hypertensive Fawn-hooded (FHH) rat that determine susceptibility of a kidney to develop damage, independent from loci coding for

hypertension.[13] In contrast to the susceptible FHH kidney, the August x Copenhagen-Irish (ACI) rat strain was resistant to develop hypertension-induced renal damage.[14] (FHHxACI)F_1 rats were also quite resistant to renal damage indicating the resist nature of this trait.[13,15] However, in the previous studies the kidneys functioned in different environments. Thus the question remains whether these differences in susceptibility to CTD are intrinsic to the kidney or are related to metabolic, hormonal or other factors, such as the level of blood pressure.

To test the hypothesis that in a normotensive environment, the susceptibility of the donor kidney to develop CTD is strain-dependent, we transplanted three types of donor kidneys, the FHH, ACI and the F_1 of both strains into a histocompatible, normotensive F_1 recipient. In this setting, the donor kidneys were exposed to the same blood pressure profile, metabolic and hormonal environment.

Materials and methods

Animals

Inbred male rats, 16 weeks old and weighing 260 to 350 g at the start of the study, were used. Hypertensive FHH and normotensive ACI strains and (FHH x ACI) F_1 were derived from our breeding colony, maintained at the Erasmus University Medical School. 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*.

Kidney grafting

The left donor kidney was flushed *in situ* with cold phosphate buffered saline (4 °C), removed, cooled, and heterotopically positioned in the recipient. The donor renal artery and vein were anastomosed with 9-0 Prolene end-to-side to the recipient's infrarenal aorta and caval vein, respectively. The total ischemic time was less than 45 minutes. The donor and recipient ureter were anastomosed end-to-end with 10-0 Prolene sutures. The 2 native kidneys of the recipient were removed: the homolateral kidney at time of transplantation and the contralateral kidney 1 week after transplantation.

Experimental groups

To study the effect of the donor kidney's susceptibility to develop CTD, three strains of kidney donors were used: FHH (n = 6), ACI (n = 7), and (ACI x FHH) F_1 (n = 6). The (ACI x FHH) F_1 rats were used as recipients. Weight-matched unilaterally nephrectomised (UNx) FHH (n = 8), ACI (n = 10) and F_1 (n = 9) rats served as controls.

Functional measurements

Body weight, systolic blood pressure (SBP) and urinary albumin excretion (UaV) were determined before transplantation and at week 8 and 16 postoperatively. Systolic blood pressure was measured by tail-cuff plethysmography (IITC Life Science, Woodland Hills, CA) in awake, restrained animals. Twenty-four-hour urine was collected by placing the rats in metabolic cages. Actual measurements were done on 2 consecutive days for assessment of albumin excretion, which was measured with bromocresol green (Merck, Darmstadt, Germany). At week 16 post-transplantation, the plasma creatinine was analysed with the Jaffé method without deproteinization.

Tissue processing

Kidneys and hearts were harvested under ether anesthesia and weighed. Recipients without complications of grafting (pyelonephritis or hydronephrosis) were included in the study. The kidneys were fixed by immersion for 48 hr in a 3.6% buffered formaldehyde solution after longitudinal bisection, and embedded in paraffin for histopathological studies. Paraffin sections (3 µm) were stained with periodic acid Schiff and hematoxylin/eosin counterstain.

The kidney sections were microscopically examined for the presence of focal glomerulosclerosis (FGS). In a single kidney section, 50 glomeruli were examined in the inner and outer cortical region and the number of sclerotic glomeruli was determined. FGS was defined as previously described.[14] In addition, kidney sections were evaluated for chronic transplant dysfunction using the BANFF-criteria.[16] Briefly, glomerulopathy - determined in nonsclerotic glomeruli -, interstitial fibrosis, tubular atrophy and intimal thickening were separately determined with a score ranging from 0 = normal, 1 = up to 25% affected, 2 = moderate, affecting 25-50%, and 3 = more than 50% changes. The sum of the individual scores of the 4 criteria is given as the total BANFF score. Sections were evaluated as 'blind' to group.

Statistical analysis

All statistical analyses were performed using one way analysis of variance followed by the Student-Newman-Keuls test. The results are expressed as mean ± SD in the text and tables, and as mean ± SEM in the figures; probability of $p < 0.05$ was accepted as significant.

Results

The susceptibility for CTD was studied by using 3 different types of donors, transplanted into the histocompatible, normotensive F_1 recipient.

F_1 transplants

The SBP of F_1 kidney transplants did not differ from that in UNx controls, both having a stable SBP during the follow-up (Figure 1). Heart and kidney weight were identical (Table 1). A negative impact of the transplantation procedure on the development of CTD could not be observed. F_1 transplants had a stable renal function. UaV varied between 10 to 16 mg/day during follow-up, and did not

		week 0			*week 16*		
Donor-Rec	n	BW-donor	KW-donor	BW-rec	BW	KW	HW/100 g
F_1 - F_1	6	332 ± 22	1375 ± 108	340 ±27	408 ± 22	1998± 22	232 ± 9
ACI - F_1	7	266 ± 11*	996 ± 59*	334 ±20	397 ± 36	2131 ±169	240 ± 31
FHH-F_1	6	331 ± 17	1349 ± 190	342±26	402 ± 13	1955 ±136	251 ± 16
F_1 UNx	9	344 ± 40	1380 ± 86		388 ± 47	1920 ±118	254 ± 14
ACI UNx	10	271 ± 19*	1008± 70†		289 ± 25	1387 ±84	248 ± 10
FHH UNx	8	351 ± 24	1387 ± 197		375 ± 30	2475 ±487	285 ± 19#

Table 1. Body, kidney and heart (absolute or relative) weights at time of transplantation and at autopsy. Rec, recipient; UNx, unilaterally nephrectomized; BW, body weight in g; KW, wet kidney weight in mg; HW/100g, wet heart weight/100g BW. Values are given as mean ± SD. * = $p < 0.05$ ACI body weight vs F_1 and FHH body weight; † = $p < 0.05$ ACI kidney weight vs F_1 and FHH kidney weight; # = $p < 0.05$ FHH UNx vs FHH-F_1

differ from F_1-UNx (Figure 2). They also demonstrated a similar degree of FGS and BANFF score as their UNx controls (Figure 3).

ACI transplants

Transplanting an ACI kidney into the F_1 did not influence the recipient's SBP: At 16 weeks, the blood pressure did not differ from that in F_1-F_1 combination, 123 ± 8 vs 121 ± 9 mm Hg, respectively (Figure 1). In addition to the SBP measurements, the relative heart weight was equal in both groups (Table 1). At the end of the experiment, the relative and absolute kidney weight of ACI and F_1 transplants was similar. Because the absolute kidney weight of the ACI donors at time of transplantation was significantly lower than that of F_1 donors, renal hypertrophy was most profound in ACI transplants (Table 1).

Apart from the blood pressure profile and renal mass, function determined by UaV (Figure 2) and plasma creatinine (Table 2) of transplanted ACI kidneys did not differ from that of F_1 transplants. Both ACI and F_1 kidney grafts

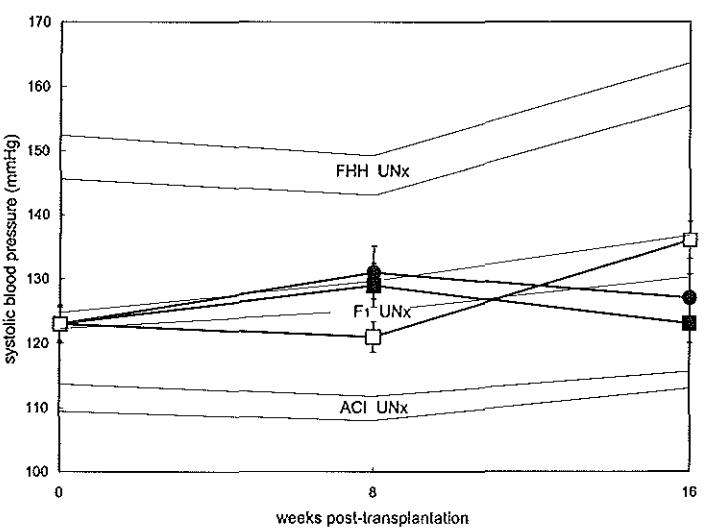

Figure 1. Tail cuff systolic blood pressure (SBP) in millimeters of mercury in awake (ACI x FHH) F_1 recipients with transplanted kidneys from the hypertensive Fawn-hooded (FHH)(□), the normotensive ACI(■) strain and from the F_1 (●) of both strains and in awake unilaterally nephrectomized (UNx) FHH, ACI and F_1 rats. Values are given as mean ± SEM. * = $p < 0.05$ vs FHH UNx, at 8 and 16 weeks post-transplantation

demonstrated a minimal degree of damage: 14 ± 7 and 10 ± 6 % sclerosed glomeruli. The BANFF score was also identical in ACI and F_1 transplants, and was mainly based on some glomerulopathy and tubular atrophy (Figure 3).

ACI transplants versus ACI-UNx

The SBP of ACI transplants was significantly higher at 8 weeks than that of ACI-UNx, 129 ± 9 vs 110 ± 6 mm Hg, respectively. However, at 16 weeks no significant difference in SBP could be detected anymore (123 ± 8 and 115 ± 6 mm Hg).

Renal function (Figure 2, Table 2) did not change after transplantation: At week 16, UaV of ACI transplants was 24 ± 12 mg/24hr, which was not significantly different from 8 ± 5 mg/24hr of ACI-UNx. There was no difference in degree of FGS, glomerulopathy, tubular atrophy or interstitial fibrosis in both groups (Figure 3).

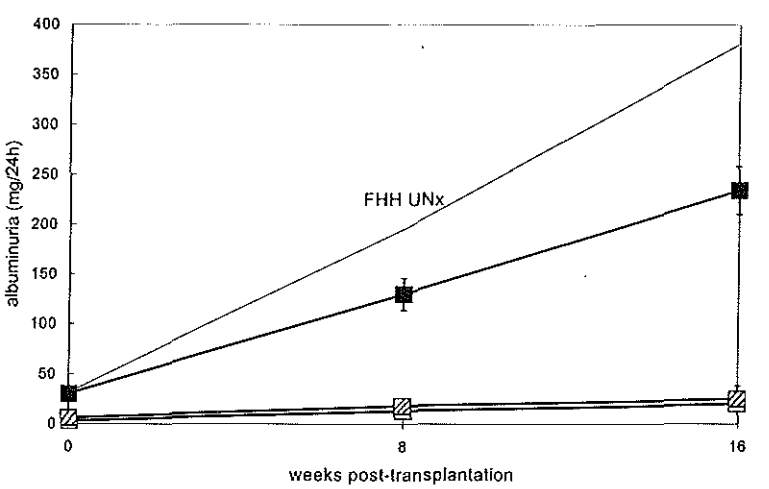

Figure 2. Urinary albumin excretion in mg/24h in (ACI x FHH) F_1 recipients with the Fawn-hooded (FHH)(■), the (ACI)(▨) strain and the F_1 (□)of both strains as donor and in unilaterally nephrectomized (UNx) FHH, ACI and F_1 rats. Values are given as mean ± SEM. * = $p < 0.05$ vs F_1-F_1 and FHH-UNx, at 8 and 16 weeks post-transplantation.

FHH transplants

Grafting an FHH kidney to the F_1 did not change the SBP of the F_1 recipient (Figure 1). Similarly, the relative heart weights of both groups were equal (table 1). The absolute and relative kidney weight of both groups was similar, both at time of transplantation and at the end of the experiment (Table 1). Despite comparable blood pressure profiles and renal mass, the severity of CTD differed significantly between FHH and F_1 transplants. Renal function was impaired. FHH kidney grafts excreted significantly more UaV than F_1 transplants, 234 ± 58 vs 25 ± 30 mg/24 hr, respectively, at week 16 (Figure 2). Plasma creatinine, a less sensitive marker, did not differ between both groups (Table 2). The histomorphology also demonstrated a striking difference between both groups. Whereas F_1 transplants had minimal degree of damage, FHH grafts demonstrated moderate FGS (10 ± 6 vs 39 ± 12%, respectively; $p < 0.05$).

Evaluation of renal damage before transplantation demonstrated that kidneys from FHH, ACI and F_1 had minimal FGS at the time of transplantation, although FHH kidneys with 8 ± 5% had significantly more damage than ACI and F_1 kidneys with 1 ± 1 and 2 ± 2%, respectively.

	F_1-F_1	ACI-F_1	FHH-F_1	F_1 UNx	ACI UNx	FHH UNx
Creatinine (µmol/L)	59 ± 6	60 ± 5	74 ± 17*	60 ± 8	66 ± 7	102 ± 50

Table 2. Plasma creatinine levels in kidney transplants at week 16. * = $p < 0.05$ vs FHH UNx.

FHH transplants versus FHH UNx

Comparing the transplanted FHH kidney to the hypertensive FHH UNx, the SBP and the relative heart weight of the FHH-F_1 were significantly lower (Figure 1, Table 1). The rise of UaV (Figure 2) in FHH transplants was significantly attenuated. At week 16, UaV was 234 ± 58 compared to 379 ± 86 mg/24 hr in the FHH-UNx. In addition, the transplanted FHH kidney had significantly lower plasma creatinine levels compared to FHH-UNx (Table 2). Besides a less impaired function, transplanting an FHH kidney into the F_1 also resulted in a

significantly lower incidence of FGS and lower BANFF score with significantly less glomerulopathy, tubular atrophy, and interstitial fibrosis compared to that observed in FHH-UNx, whereas the degree of intimal hyperplasia did not differ between both groups (Figure 3).

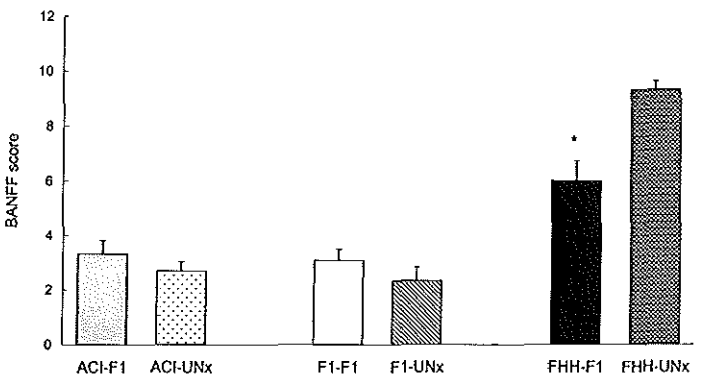

Figure 4a. The histopathological degree of chronic transplant dysfuction according to the BANFF-criteria in kidneys of Fawn-hooded (FHH), ACI and the F_1 of both strains transplanted into the histocompatible F_1 recipient, and in unilaterally nephrectomized (UNx) kidneys of the 3 strains. The 4 different criteria - 1) glomerulopathy in nonsclerotic glomeruli, 2) tubular atrophy, 3) interstitial fibrosis and 4) intimal hyperplasia were separately analyzed as described in the Methods, and the sum of the individual scores of the 4 criteria is given. Values are given as mean ± SEM. * = $p<0.05$ vs F_1-F_1 and FHH UNx.

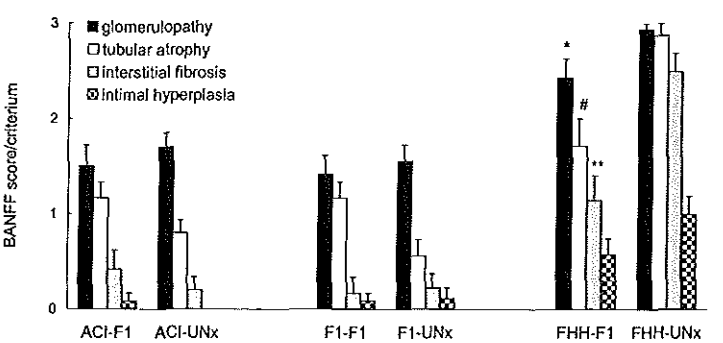

Figure 4b. The histopathological degree of chronic transplant dysfunction differentiated by the 4 criteria: 1) glomerulopathy in nonsclerotic glomeruli, 2) tubular atrophy, 3) interstitial fibrosis and 4) intimal hyperplasia. Values are given as mean ± SEM. *, #, ** = $p<0.05$ vs F_1-F_1 and FHH UNx.

Discussion

To test the hypothesis that in a normotensive environment, the susceptibility of the donor kidney to develop CTD is under genetic control, we transplanted kidneys of two strains (FHH and ACI) and their F_1 into the normotensive, histocompatible F_1 recipient. In this setting, the donor kidneys were exposed to the same blood pressure profile, metabolic and hormonal environment.

The main finding of this study was that genetic factors of the donor kidney contribute to the development of CTD. Transplanting an ACI kidney into a normotensive F_1 rat resulted in a normal renal function that was comparable to that of F_1 transplants. In contrast, kidneys from hypertensive FHH donors transplanted to F_1 demonstrated a significant rise in albuminuria and glomerulosclerosis compared to ACI or F_1 transplants, whereas the systolic blood pressure was similar in the three transplant groups. The normotensive status of the F_1 with different donor kidneys is directly based on tail-cuff SBP measurements at two time points after transplantation. Although the sensitivity of the indirect measurement of SBP is restricted, even with measuring it five times, additional evidence for normotensive profiles comes from the finding that absolute and relative heart weights were equal. Thus, factors other than hypertension have to be related to the differences in susceptibility for CTD between the FHH and ACI rats.

The ACI donor weighed less than the F_1 recipient at time of transplantation, hence the ACI kidney was transplanted into an unmatched recipient. At the end of the follow-up, the kidney weight of ACI transplants was similar to that of F_1 transplants. Even with this compensatory hypertrophy to fully adapt to the heavier recipient, the ACI did not demonstrate features of impaired function or altered morphology, which emphasizes its genetic resistance to develop renal damage. On the other hand, the susceptibility of FHH kidneys was not related to differences in relative or absolute kidney weight since FHH and F_1 had equal values. Furthermore, the increased susceptibility of the FHH could not be attributed to a difference in number of glomeruli. At a age of 13 weeks FHH and ACI kidneys both have 33.000 ± 3800 glomeruli (Provoost AP, van Aken M, unpublished data).[17]

From previous studies in the FHH rat, we know that the susceptibility of the FHH to develop glomerulosclerosis is associated with elevated intraglomerular pressure. Simons *et al.* demonstrated glomerular capillary hypertension in

moderately hypertensive FHH rats,[18] which suggests that the autoregulation of the kidney does not function properly. Therefore, it might be that the systolic blood pressure of the F_1 was still too high for the FHH kidney. Previously, we demonstrated in FHH-UNx that after decreasing the SBP to 115 mm Hg with angiotensin converting enzyme inhibitors the incidence of glomerulosclerosis was lower than we observed in the transplanted FHH kidneys.[19] On the other hand, decreasing the SBP of the FHH UNx to the level of the FHH transplants by irbesartan caused much less glomerulosclerosis than observed in our FHH transplants.[20] However, such pharmalogic intervention reduces SBP, but additional effects directly protecting the kidney cannot be excluded.

The degree of CTD could also be related to a genetic susceptibility of kidneys to ischemia-reperfusion injury. Such early events have been demonstrated to induce or accelerate CTD.[12,21] Because transplantation of an ACI or F_1 donor kidney into the F_1 recipient did not affect urinary excretion of albumin or renal morphology compared to UNx controls, we are able to transplant kidneys without impairing renal function. In addition, during a 1-year period our transplantation technique does not lead to functional and morphological changes in syngeneic BN kidneys.[12] Therefore, the renal impairment of the FHH transplants could reflect susceptibility for ischemia-reperfusion injury. Using the same transplantation technique, kidneys from the WAG/Ro strain are much more susceptible for damage than BN kidneys. WAG/Ro kidneys demonstrate a delayed function, whereas BN kidneys produce urine immediately after anastomosing the renal vessels.[22] To verify this hypothesis, FHH should be transplanted into the FHH. However, surgery with vascular anastomosis is technically impossible because of the genetic bleeding disorder of the FHH rat. Another approach to resolve this question is by temporary clamping the renal artery in unilateral nephrectomized FHH and ACI, both treated with antihypertensive drugs.

One compromising factor in unraveling the mechanism of susceptibility could be the donor kidney morphological status. At time of transplant, FHH kidneys showed somewhat more glomerulosclerosis than ACI kidneys, which could influence the outcome. To exclude this, treatment with antihypertensive drugs in FHH and ACI rats early after birth until transplantation could prevent any hypertension-induced renal damage.

The present study confirms previous findings[14] that hypertension has to be associated to the genetic susceptibility of the FHH kidney, as damage could be

attenuated by transplanting it into a normotensive F_1 recipient. This result supports the idea that a proper control of blood pressure in kidney transplant patients will reduce the progression of CTD, as demonstrated earlier by Paul *et al.* in the Fisher to Lewis rat transplantation model.[23]

Besides the observed differences in susceptibility to CTD, this transplantation study confirms phenotypically the previous finding that susceptibility for renal disease and hypertension are under independent genetic control in the FHH rat.[13] Because the F_1 recipients remained normotensive after transplantation, the moderate hypertension of the FHH rat strain did not travel with the kidney. This makes the FHH strain a hypertensive rat strain in which hypertension does not appear to travel with the kidney. Experimental studies with genetically hypertensive rat strains as the Milan hypertensive,[24] the Dahl-S hypertensive,[25,26] and the SHR strain[27] demonstrated that hypertension is transferable. Also, some clinical studies suggest that hypertension of the donor could contribute to post-transplant hypertension in recipients.[28,29] However, results of our study clearly demonstrate that hereditary hypertension does not always have its origin in the kidney.

In summary, the genetic susceptibility of the donor kidney is important in the development of chronic transplant dysfunction. The responsible genes for this susceptibility and the pathophysiological mechanisms are currently under investigation.

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8

Functional, histological, and inflammatory changes in chronically rejecting small bowel transplants

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Submitted for publication

Summary

Background: Our aim was to develop a model of chronic rejection (CR) in small bowel allografts, and to study the changes occurring in these grafts.
Methods: Small bowel transplantation was performed using the DA to AS rat strain combination. Short-term (5 mg/kg intramuscular, from days -2 to +9), or long-term cyclosporine treatment (5 mg/kg, 3 times a week until day 50) was given to prevent acute rejection. Controls were untreated allografts, DA isografts with and without cyclosporine, and normal DA and AS rats. They were followed for 50 and 100 days after transplantation.
Results: Recipients of a syngeneic graft lost weight during the first week after transplantation, but started to regain weight and kept growing thereafter. Histology showed normal bowel architecture with normal mesenteric lymph nodes and Peyers patches. Vigorous acute rejection occurred in the untreated allografts. Animals had persistent weight loss, and were killed between 6 to 13 days after transplantation. No clinical signs of graft-versus-host disease were seen. Histology showed end-stage acute rejection. In both cyclosporine treated allografted groups the postoperative course was as in the isografted animals. However, all animals had histologic signs of CR by 50 and 100 days after transplantation. Changes were most prominent in the mesentery. Serositis with increased vascularity, inflammation with sclerosis, and patchy myointimal proliferation with endothelialitis of the mesenteric vessels were found. Changes in the bowel were patchy and included some thickening of the muscle coat, crypt hyperplasia, scattered necrotic cells in the crypts, slight blunting of villi and loss of goblet cells. Infiltrating cells in the mesentery and bowel consisted mainly of $CD4^+$ cells, $CD8^+$ T-cells and monocytes/macrophages. Lactulose-mannitol urinary excretion ratio was significantly increased in short-term cyclosporine treated allografts at days 50 and 100 posttransplant. Serum albumin levels were significantly lowered in this group at both time points examined.
Conclusions: We have developed two models in which CR occurs after small bowel transplantation. Long-term cyclosporine treatment delayed the development of CR, since functional abnormalities were only seen in the animals that were treated with short-term cyclosporine.

Introduction

Small bowel transplantation (SBT) is potentially an improved treatment modality for patients suffering from irreversible short-bowel syndrome, who are currently fed using total parenteral nutrition (TPN). In specialized centers, SBT has evolved over the last decade from a cumbersome procedure with unacceptably high morbidity and mortality to a more acceptable therapy for those with permanent intestinal failure. Patients that qualify for SBT are those with irreversible dependency on TPN and a poor long-term prognosis related to progressive difficulties with the administration of TPN (venous access), catheter associated sepsis, and liver failure.[1,2]

A major problem hampering the long-term success of SBT is the vigorous acute rejection, requiring high doses of immunosuppression. A major improvement in the results of SBT came in the early 1990-ies when the powerful immunosuppressant tacrolimus replaced cyclosporine. Although the outcome of SBT is still mainly determined by acute rejection and lethal infections, the long-term results are nevertheless improving.[1] Because of this, new problems that arise in the longer term are encountered. Of these, dysmotility, eating disorders, post transplant lymphoproliferative disease, late acute rejection episodes and chronic rejection (CR) are of special concern.

Little is currently known about CR following SBT, although some experimental and clinical data show that CR may develop after SBT.[3,4] The clinical characteristics reported on CR are diarrhoea and weight loss. Histologically, total villous atrophy, apoptosis of crypt cells with inflammatory infiltrate, and arteriosclerosis of the mesenteric vessels are described.[5,6]

The aim of the present study was to develop a model in which CR of the small bowel allograft would develop in a reproducible manner that would allow us to study the changes occurring in those grafts.

Materials and methods

Animals

Inbred adult male Dark Agouti (DA) ($RT1^{av1}$) and Albino Surgery (AS)($RT1^{1}$) rats were obtained from Monash Animal Services, Victoria, Australia. All experimental procedures involving animals conformed to the National Health

and Medical Research Council Code of Practice and were approved by Monash Medical Centre Animal Ethics Committee B and the Monash Standing Committee on Ethics in Animal Experimentation.

Small bowel transplantation

SBT was performed as described previously.[7] In brief, the total small bowel, from the Ligament of Treitz to the terminal ileum was transplanted on a vascular pedicle consisting of the superior mesenteric artery and portal vein. In the recipient, end-to-side anastomoses were made between the recipient infra-renal aorta and donor superior mesenteric artery, and recipient caval vein and donor portal vein. After resection of the recipient small bowel, the graft and recipient remnant bowel were anastomosed end-to-end.

Experimental design

The following groups were studied: 1) DA to DA, no treatment, 2) DA to DA, long-term cyclosporin (lCsA), 3) DA to AS, no treatment, 4) DA to AS, short-term cyclosporine (sCsA), and 5) DA to AS, long-term CsA. sCsA treatment consisted of 5 mg/kg intramuscularly from day -2 to +9 relative to transplantation. lCsA treatment consisted of 5 mg/kg intramuscularly three times a week until day 50. Normal DA or AS rats were used as controls. Animals were killed 50 and 100 days after transplantation, or when they demonstrated 30% weight loss.

Functional parameters

Serum albumin was measured using the bromocresol green method on a Cobias bio autoanalyser. Results are given as mean ± sd in g/l. Lactulose mannitol urinary excretion test: Animals were fasted during the day. 400 mg/kg D-lactulose and 100 mg/kg of D-mannitol were dissolved in 1 mL of water. This solution was given orally by gavage. Each animal was placed in a metabolic cage and urine was collected overnight. The amount of urine produced was recorded, and samples were stored at -20 °C until analysis. Both lactulose[8] and mannitol [9] concentrations (mmol/l) were measured colorimetrically on a Cobias Bio Autoanalyser. The results are expressed as mean ± SEM lactulose: mannitol ratio.

Macroscopic appearance of the graft

The small bowel graft, its mesentery, the Peyers patches, mesenteric lymph nodes, and spleen were inspected for signs of CR at the time of organ retrieval.

Samples of the graft were prepared for histology and immunohistochemistry.

Histology

Samples of graft ileum were fixed in 10% neutral formalin. After dehydrating and embedding in paraffin, 3-4 µm thick sections were cut and stained with hematoxylin and eosin (H&E). They were scored by two observers. The incidence of the following features was recorded for each of nine high power fields (400 X); Blunting of villi and loss of goblet cells, crypt hyperplasia and crypt cell necrosis muscularis thickening, cellular infiltration, vascular involvement (ie myointimal proliferation, endothelialitis, adherence of leucocytes to the endothelium, and perivascular infiltration), and sclerosis in the mesentery. Scores of 0, 1, 2, or 3 were assigned if features were observed in 0, 1/3, 2/3, or all fields respectively. Results for each feature were added up, and histologic grades were assigned as follows: 0 points = none (0), 1-3 points = very mild (grade 1), 4-6 points = mild (grade 2), 7-9 points = moderate (grade 3), and 10-12 points = severe (grade 4).

Immunohistochemistry

Tissues were embedded in OCT and snap frozen. Cryostat sections of 7 µm thick were cut. These sections were fixed in PLP, and stained using a three-layer peroxidase technique.[10] In brief, sections were pre incubated for 10 minutes in 10% Normal Swine Serum (NSS; Dako, Christchurch, New Zealand) / 10% Fetal Calf Serum (FCS) in phosphate buffered saline (PBS) with 0.01% NaN_3 (Az) to avoid non-specific binding of the primary antibodies. Slides were drained, and incubated at room temperature for 1 hour with primary mouse antibody diluted in 1% NSS / 1% FCS with 0.01% Az. They were washed 1 x in PBS for 5 minutes, taken through graded alcohols, and incubated at 4 °Celcius for 10 minutes in methanol / 0.03% H_2O_2 to block endogenous peroxidase activity. Thereafter they were reversed through graded alcohols and washed 2x in PBS, Rabbit anti-mouse-peroxidase conjugate (Dako), was applied and incubated for 30 minutes at room temperature.

After washing 1 x in PBS, the third step, swine anti-rabbit-peroxidase conjugate (Dako), was applied and incubated for 30 minutes at room temperature. Sections were then washed in PBS 2 x, and colour was developed using metal-enhanced DAB substrate (Pierce-Rockford). Finally, slides were counterstained wit Harris' haematoxylin, dehydrated, and covered using glass slips.

The following mouse-anti-rat monoclonal antibodies were used: OX-1 (CD45),

OX-8 (CD8), W3/25 (CD4), OX-6 (MHC class II), OX-33 (B-cells), ED-1 (monocytes, free and tissue macrophages), ED-2 (tissue macrophages), ED-3 (lymphoid macrophages) (ED-1, 2, and 3 are a kind gift of dr C.D. Dijkstra, Amsterdam, The Netherlands), and 3.2.3. (NK cells).

The %-area OX-1 positive cells was measured using a "Video Pro" image analysis system. OX-8, W3/25, ED-1, OX-33 and 3.2.3 were counted using a graticule at a magnification of 400 X. Six separate fields were measured corresponding to an area of 0.1 mm^2. For the submucosa, an area corresponding to 0.02 mm^2 was measured and then multiplied by 5. The other markers were scored semiquantitatively.

Statistics

Statistical analysis of the nutritional parameters, histology and immunohistochemistry was done with the Kruskal Wallis analysis of variance, followed by a Mann-Whitney U-test, using SPSS for Windows. A p-value <0.05 was considered statistically significant.

Results

Postoperative recovery

Recipients of a syngeneic graft (groups 1 and 2) lost weight during the first week post transplant, but started to regain weight and kept growing thereafter. Untreated allografts demonstrated vigorous acute rejection. Animals had persistent weight loss and developed diarrhoea from day 4 onwards. They were killed between days 6 and 13 after transplantation. No signs of graft-versus-host disease were seen. Histopathological analysis showed end stage acute rejection.

CsA treated allografts (groups 4 and 5) seemed to lag behind in growth. No statistically significant differences between allografted, and syngeneically transplanted animals were demonstrable however, since the variation between the animals in the different groups was considerable (Figure 1). Since no diarrhoea or weight loss was observed at the time points studied these models may be considered as representing subclinical CR.

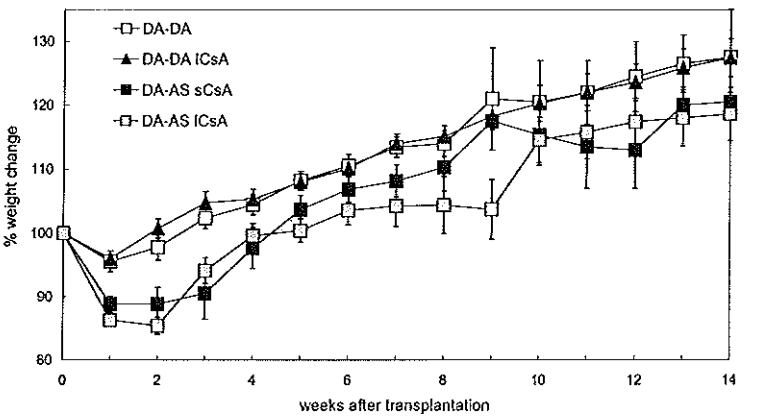


Figure 1. Weight changes after small bowel transplantation

Nutritional parameters

Serum albumin levels were significantly lowered in sCsA treated animals at both time points as compared to normal AS rats (28.1±4.9 and 27.2±2.3 vs. 34.8±0.8 g/l, p=0.02 and p=0.006, respectively). At day 50, the lactulose-mannitol urinary excretion ratio in sCsA allografts was significantly higher than that in lCsA allografts and normal DA controls (Figure 2). Thereafter, the ratio further increased. Hundred days after engraftment, the sCsA allografts had significantly higher ratios than the lCsA treated ones, isografts and normal DA controls. There were no significant differences between control rats and syngeneically transplanted groups. CsA treatment in the isografts did not affect the excretion of both urinary lactulose and mannitol.

The lactulose-mannitol excretion significantly correlated to the histological degree of chronic rejection (R=0.35, p = 0.047, n = 33).

Macroscopic appearance of the grafts

Fifty and 100 days after transplantation isografts showed a normal macroscopic bowel architecture with normal mesenteric lymph nodes and Peyers patches. Signs of CR were observed in 67% of sCsA treated animals and 33% of lCsA treated animals by 50 days, and all sCsA and 67% of lCsA animals by 100 days post transplant.

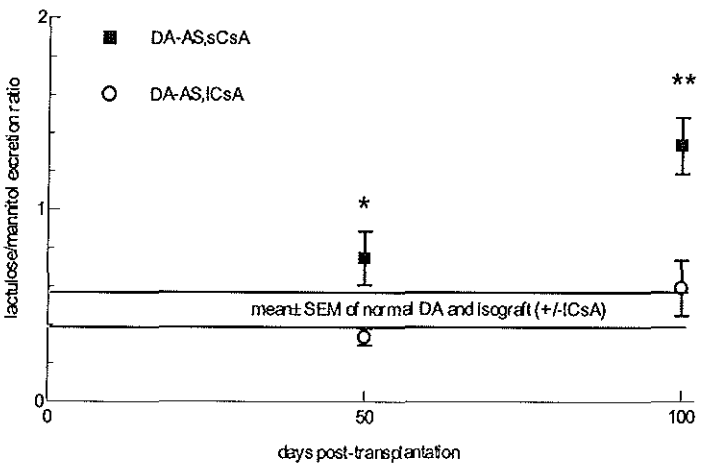


Figure 2. Intestinal function of small bowel transplants was measured by the urinary excretion of orally administered lactulose and mannitol. The ratio lactulose / mannitol was calculated and results are expressed as mean ± SD. ** =p <0.05 vs DA-AS,lCsA and DA-DA at the same timepoint, and vs DA-AS,sCsA at day 50; * =p <0.02 vs DA-AS,lCsA and DA at the same timepoint. Normal DA, DA-DA +/- lCsA did not differ, and are depicted as 'normal range bar'.

Macroscopic signs were: sclerosis and enlargement of the MLN, sclerosis of the mesentery and serosa, thickening of the bowel wall, splenomegaly, and enlargement of the cecum (both 1.5-3 times their normal size). In most allografts the Peyers patches were atrophied, sometimes to a degree that they were no longer visible. In other allografts Peyers patches had become enlarged and hemorrhagic. In advanced CR, the MLN had become a large, sclerotic and sometimes hemorrhagic mass. The mesentery and serosa were rigid due to the extensive sclerosis. None of these abnormalities were found in the syngeneic groups.

Histology

At 50 days post transplant, a pericapillary infiltrate was usually the first indication of CR (Figure 3). Other early features were cellular depletion and active infiltration of the graft MLN. Serositis with increased vascularity, florid mesenteric inflammation with sclerosis and patchy myointimal proliferation with endothelialitis of the mesenteric vessels were found later in the course of

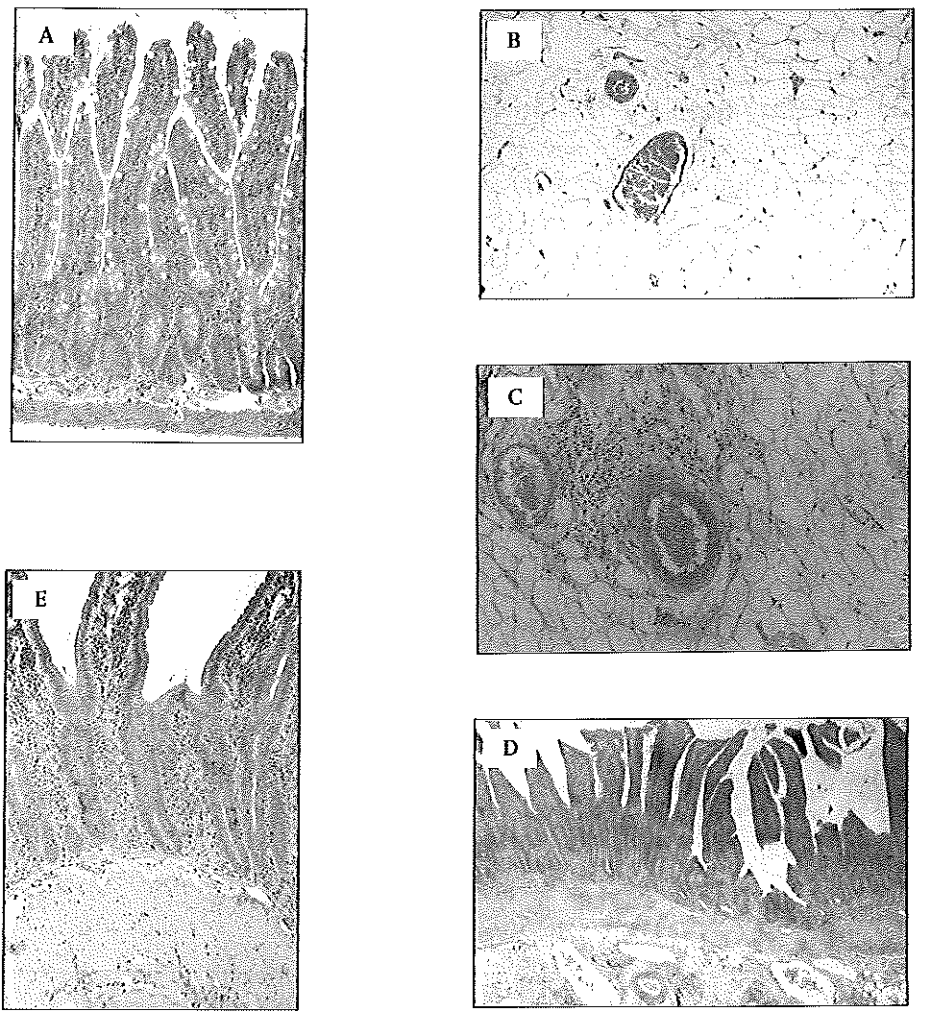


Figure 3.
a) DA to DA isograft 100 days post transplant showing normal bowel architecture. b) Mesentery of the graft shown in fig. 3a c) Pericapillary infiltrate in the mesentery of a long-term cyclosporine treated allograft at day 50 posttransplant with mild chronic rejection. d) Allograft treated with long-term cyclosporine at day 100 post transplant showing moderate chronic rejection with blunting of villi, loss of goblet cells, crypt hyperplasia, hypertrophy of the muscle coat, and sclerosis of the mesentery. e)High power magnification of an allograft treated with short-term cyclosporine at day 50 post transplant showing moderate chronic rejection with blunting of villi, loss of goblet cells, crypt hyperplasia and thickening of the muscularis.

CR. Changes in the bowel were patchy, and included some thickening of the muscle coat, crypt hyperplasia, scattered necrotic cells in the crypts, sclerosis between the crypts, slight blunting of the villi and loss of goblet cells (Figure 3). Apart from an increase in mononuclear cells, the lamina propria between the crypts also contained mast cells and eosinophils. The degree of CR could vary from very mild to moderate within a group (Table 1a). At day 50, lCsA treated animals had significantly less chronic damage compared to sCsA treated animals (1.3±0.5 vs. 2.8±0.5, p=0.001)(Table 1b). However, this difference had disappeared by day 100 after transplant.None or very mild changes were seen in the syngeneically transplanted grafts (Figure 3).

	Short-term CsA		*Long-term CsA*	
Grade↓ /day→	day 50 (n=9)	day 100 (n=5)	day 50 (n=6)	day 100 (n=6)
none (0)	0	0	0	0
very mild (1)	0	20	67	17
mild (2)	33	40	33	50
moderate (3)	67	40	0	33
severe (4)	0	0	0	0

Table 1a. Histopathological analysis of chronic rejection after small bowel transplantation. Percentage of animals per group with a chronic rejection grade. Histopathological chronic rejection grades were assigned according to the severity of the lesions (see: Materials and methods).

Group	Histopathological rejection grade (mean ± sd)
short-term CsA, day 50	2.7 ± 0.5
short-term CsA, day 100	2.2 ± 0.8
long-term CsA, day 50	1.3 ± 0.5 *
long-term CsA, day 100	2.2 ± 0.7

Table 1b. Chronic rejection grades after small bowel transplantation. Histopathologic rejection grades were assigned according to the severity of the lesions (see materials and methods).*: short-term CsA vs. long-term CsA at day 50, p=0.001.

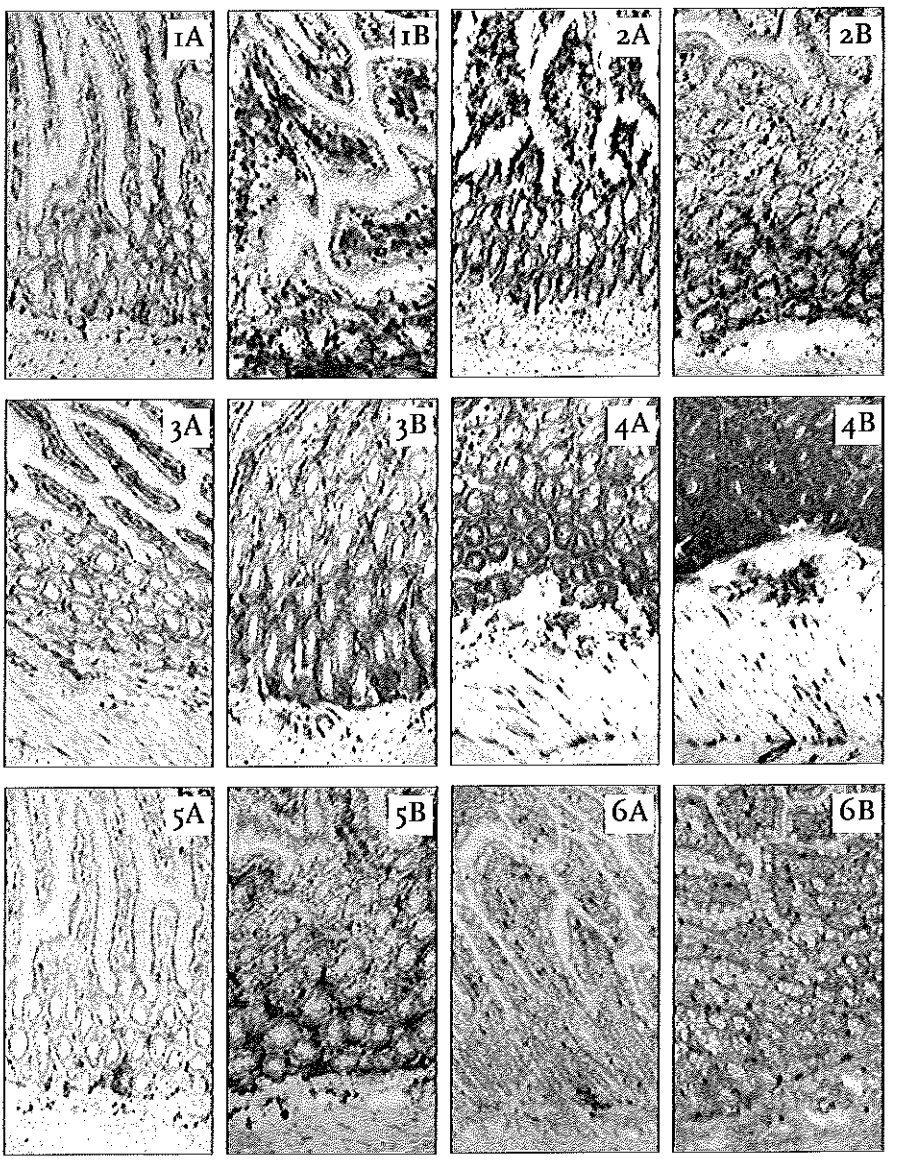
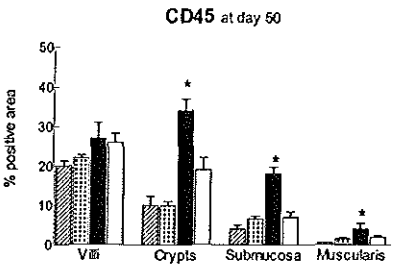
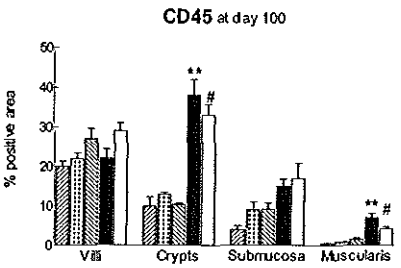
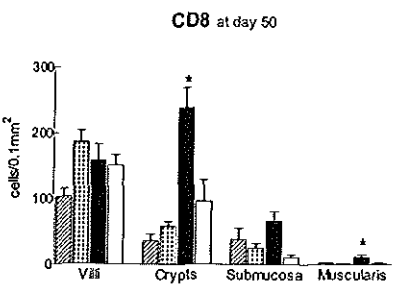
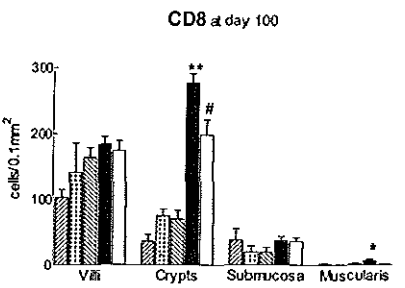


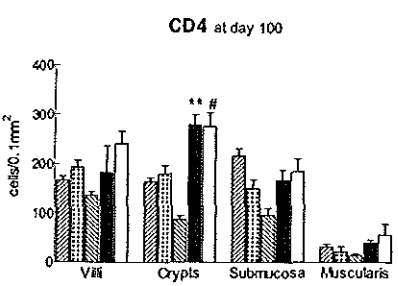
Figure 4. Distribution of 1: CD45 (OX-1), 2: CD8 (OX-8), 3: CD4 (OX-4), 4: MHC class II (OX-6), 5: macrophages (ED-1), and 6: NK cells (323) in isografts (a) and allografts (b) 100 days post transplantation.


CD45 at day 50
% positive area
Villi
Crypts
Submucosa
Muscularis


CD45 at day 100
% positive area
Villi
Crypts
Submucosa
Muscularis


CD8 at day 50
cells/0.1mm2
Villi
Crypts
Submucosa
Muscularis


CD8 at day 100
cells/0.1mm2
Villi
Crypts
Submucosa
Muscularis


CD4 at day 100
cells/0.1mm2
Villi
Crypts
Submucosa
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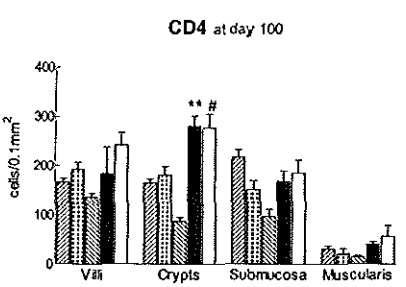
CD4 at day 100
cells/0.1mm2
Villi
Crypts
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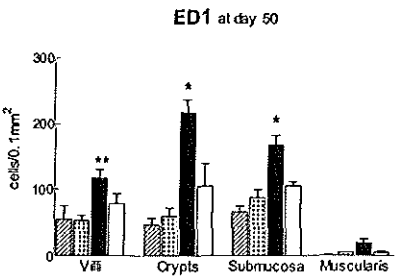
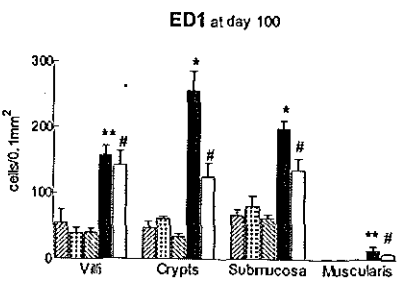
Muscularis


ED1 at day 50
cells/0.1mm2
Villi
Crypts
Submucosa
Muscularis


ED1 at day 100
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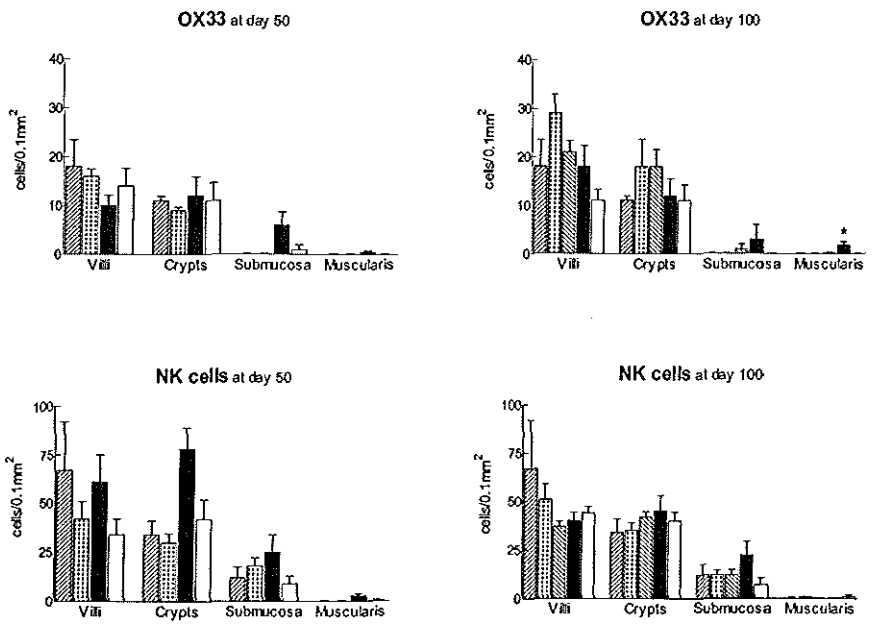

Figure 5. Cell infiltration in normal DA (▨), isografted (▦),isografted with CsA (▧) and allografted bowel(sCsA: ■ ; lCsA: □).The increased infiltration by CD45+ leucocytes in the chronically rejecting allografts consisted mainly of T lymphocytes and ED1+ macrophages. CsA therapy delayed the influx of these cells in the allografts. B lymphocytes and NK cells appeared to play a minor role in chronic rejection. Isografts with or without CsA therapy had a comparable numbers of mononuclear cells as normal DA controls. Results are expressed as mean ± SEM.* = $p<0.05$ vs DA-DA and DA-AS,lCsA at the same timepoint; ** = $p<0.05$ vs DA-DA at the same time point; # = $p<0.05$ vs DA-DA,lCsA and DA-DA at the same timepoint.

Analysis of infiltrating cells

The numbers, types, and distribution of leucocytes demonstrated in the different compartments of the ileum (i.e. villi, crypts, submucosa, and muscularis) are depicted in Figures 4 and 5. As can be seen in the figures, no significant changes were detected in cell numbers in either of the isograft groups when compared to normal DA rat controls. In some isografts however, a focal infiltrate around single crypts was seen. This was never observed in non-transplanted controls. In general, in the mucosa of small bowel grafts, the highest density of cells was found at the base of the crypts, showing a gradual decrease up the crypt-villus axis.

In allografts, the number of infiltrating cells ($CD45^+$) was significantly

increased. These consisted mainly of $CD4^+$-, $CD8^+$ T-cells and ED-1^+ macrophages. Long-term CsA treatment delayed the infiltration of the allografts, since at day 50 significantly less cells were present in the lCsA than in sCsA treated animals.

$CD4^+$ cells were present in constitutively high numbers in normal ileum and isografts (Figure 4.3a). A significant increase was observed in the crypts of allografts at both time points. $CD8^+$ cells were significantly increased in the crypts and muscularis of sCsA treated allografts, and in the crypts of lCsA treated allografts at day 100. Changes in number of ED-1^+ macrophages were evident in all compartments, with a significant increase in the crypts and submucosa. In the muscle layer, which is normally devoid of ED-1^+ cells, a significant influx was observed at day 100.

The absolute number of OX-33^+ B-cells was low in all groups (Figure 5). No differences among the groups were found except for a significant increase in the muscularis of sCsA treated allografts at day 100. The number and distribution of NK cells was comparable in all groups (Figures 4.6b and 5). ED-2^+ tissue macrophages were markedly increased in the submucosa and muscularis of allografted animals. Many cells were situated at the border between the longitudinal and circular muscle layers, i.e. in close association with the myenteric plexus. Very low numbers of ED-3^+ lymphoid macrophages were seen in the crypts, submucosa, and muscularis. These numbers were comparable in all groups studied.

Discussion

Chronic rejection is the major determinant that hampers long-term graft survival of solid organ grafts.[11,12] With the improving results after seen after SBT,[1] CR will also emerge as an obstacle to long-term survival. Although its pathophysiology seems in part similar in different types of grafts, organ specific features are also apparent. Aspecific features that predominate are thickening of the intima of the blood vessels in the graft, and fibrosis.[13] Specific features are unique to the anatomy and physiology of the graft, and include tubulointerstitial damage in kidneys,[14] loss of bile ducts in liver grafts,[15] and blunting of villi in the small bowel.[3] Because we wished to study the specific pathophysiology of CR following SBT, the aim of the present study was to develop a model for CR after SBT.

The macropathological findings of our study are grossly comparable to the findings published previously by Langrehr *et al.*,[3] namely: enlargement of the MLN and involvement of the Peyers patches, fibrosis of the mesentery and in a later stage the serosa. Histological findings were quite similar also: cryptitis, blunting of villi, loss of architecture and cellular depletion of the MLN. Loss of goblet cells was not reported. In their study, however, these changes progressed rapidly after the discontinuation of CsA, whereas we found that CR had developed in all animals 50 days posttransplant, but did not rapidly progress over the next 50 days. This difference may be explained by the fact that we killed the animals at the time points of evaluation and did not take consecutive biopsies of the graft, as did Langrehr *et al.* These successive laparotomies and biopsies of the graft cause multiple episodes of wound healing with the release of growth factors that may accelerate fibrotic changes in the graft and evoke multiple adhesions.

No histological, and only very minimal inflammatory changes were found in the isografted groups. There was no difference between CsA-treated or untreated animals. This is in accordance with previous findings in syngeneic small bowel grafts,[3,15] but contrasts with recent observations in syngeneic kidney transplants where significant changes may be seen in the absence of alloantigenic stimulation,[16] and shows that the pathophysiology of CR in different organs may vary considerably.

The earliest histologic signs of CR in our small bowel transplants were loss of architecture and cellular depletion of the MLN and Peyers patches together with a pericapillary infiltrate of the mesenteric vessels. These changes were also observed in allogeneic SBT models in which no morphologic or functional changes were seen more than one year after grafting.[17] This underscores the highly immunogenic nature of these lymphoid structures and the mesentery.

Mucosal ischemia may be important in the development of CR since arteriosclerosis, which may lead to ischemia, is one of the characteristics observed in this study. The small bowel has an enormous regenerative potential after ischemic damage.[18] This regeneration starts in the crypts which respond with an increase in proliferating cells (crypt hyperplasia). These proliferating cells, which constitutively express MHC class II antigens may be primary targets for acute and chronic rejection. This may explain the high density of infiltrating cells seen in the crypts.

Rejection of the small bowel is not a generalised process. Acute rejection is patchy,[2,19] and may be easily missed when taking biopsies. Here we show that CR is also a very patchy process, in one graft both normal appearing bowel and severe

lesions may be found. This may also explain the variation found within the groups with respect to histologic score, numbers of infiltrating cells, and lactulose: mannitol excretion. Therefore, early detection of CR in human SBT will be possible only when histology is combined with immunohistochemistry and functional evaluation.

This is the first comprehensive phenotypic analysis of cells involved in chronic rejection after small bowel transplantation. The increase in $CD45^+$ leucocytes in the graft was mainly due to an increase in T-lymphocytes and ED-1^+ macrophages. These cells are also dominant in acute rejection episodes of the small bowel in man.[20,21] Pericryptic T cell infiltration preceeds histologic overt acute rejection and seems an early marker for acute rejection.[20] The focal pericryptic infiltration with $CD8^+$ cells in the present study again indicates that the crypts are an early target for both alloantigen-independent (syngeneic grafts) and alloantigen-dependent destruction. How these infiltrating cells damage the enterocytes is not clear. However, in several suspected immune-mediated small intestinal disorders crypt hyperplasia and villus atrophy are observed. It has been shown recently that activated macrophages can directly inhibit the proliferation of rat intestinal epithelial cells. Mediators produced by macrophages may be involved, and both nitric oxide and TNF-α have been show to be involved in this cytostatic effect.[22] Eosinophils and mast cells were observed in the allografts also. These cells are found in inflammatory diseases of the bowel, such as celiac disease[23] and Crohn's disease.[24] Their possible contribution to CR in our model is intriguing, and deserves further study.

In the muscle layer of allografts significant infiltration with macrophages (both ED-1^+ and ED-2^+) was seen. These cells may contribute to the thickening of the muscle wall observed, and to the loss of contractile activity reported by others.[25] This by secreting cytokines and by activating the myocytes and cells in the myenteric plexus to produce cytokines, which may exacerbate the process.

B lymphocytes and NK cells did not appear to be involved in the development of chronic rejection: There was no significant difference in the numbers of OX-33^+ B cells and $3.2.3^+$ NK cells in the different groups. This in contrast to the findings by Bauer and co-workers who observed a complete loss of B cells in the mucosa[26] and an increase of NK cells[27] of chronically rejecting small bowel grafts.

Histologic changes in the graft were accompanied by loss of graft function. Although we found no statistically significant differences in growth between iso and allografts, serum albumin was significantly lowered in the group treated

with short-term CsA. Serum albumin is an important nutritional parameter and lowered serum levels indicate that the small bowel graft was unable to normally digest and/or absorb nutrients. The lactulose-mannitol test is a widely accepted method to test intestinal permeability.[28] Lactulose is normally not absorbed by the intestinal mucosa but under pathologic conditions, it transfers paracellularly and is subsequently secreted in the urine. Mannitol is normally absorbed transcellularly, and excreted in the urine. Under pathologic conditions its absorption is impaired, and its excretion diminished. The urinary excretion ratio therefore is a sensitive measure for mucosal integrity. In our model, lactulose-mannitol ratio was significantly increased in sCsA treated allografts, which indicates a compromised epithelial integrity with transcelluar leakage. In addition, we hypothesise that the loss of goblet cells, which produce the constituents of the overlying mucus layer, results in loss of this layer. Although the role of mucus is not fully understood, there is increasing evidence that both glycoproteins and trefoil peptides present in mucus play a role in mucosal repair after injury.[29] Together, the loss of integrity of the epithelium, and the loss of goblet cells may contribute to the progression of mucosal atrophy by further impairing its capability to respond to injury.

Although it is known that CsA has a profound effect on small bowel function even in normal animals[30] the changes we found seem not attributable to CsA toxicity since function and morphology of isografts treated with CsA was normal.

Long-term CsA treatment delayed the development of CR somewhat. At 50 days posttransplant 67% of the lCsA group had very mild CR and none had moderate CR. In the sCsA group this was the reverse. However, 100 days posttransplant (i.e. 50 days without CsA in the lCsA group and 93 days in the sCsA group) this difference had disappeared. Nonetheless, the mucosal integrity in the sCsA allografts, as defined by lactulose/mannitol excretion, had further deteriorated at day 100 compared to the allografts treated with long-term CsA.

These findings show we have developed two rat models in which CR develops in a reproducible manner. Histologic and inflammatory changes are accompanied by loss of function only in short-term CsA treated animals. Clinical signs of CR were not present at the time points studied, representing subclinical CR. Long-term CsA treatment delays the development of CR, but is unable to prevent it. Further studies using this model are currently done to determine the role of growth factors in the pathophysiology of CR following intestinal transplantation.

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9

EGF and TGF-β_1 gene expression in chronically rejecting small bowel transplants

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Summary

Background: Long-term survival of small bowel transplants is hampered by chronic rejection. Epidermal growth factor (EGF) and transforming growth factor β (TGF-β) have opposing, regulatory roles in normal intestinal physiology and may be involved in the pathogenesis of chronic intestinal rejection.
Aims: To investigate the expression of EGF and TGF-β_1 in chronically rejecting small bowel transplants.
Methods: Orthotopic small bowel transplantation was performed in the allogeneic DA to AS rat combination; Cyclosporin was administered temporarily to prevent acute rejection. Controls were DA isografts and normal DA rats. PreproEGF and TGF-β_1 gene expression was evaluated by Northern blot analysis of the ileum RNA and standardized against glyceraldehyde-3-phosphate-dehydrogenase expression.
Results: Allografts demonstrated functional impairment and histological features of chronic rejection, whereas isografts appeared normal. EGF: Allografts demonstrated a significant reduction of EGF mRNA when compared to DA isografts. TGF-β_1: No significant changes were detected in TGF-β_1 expression in either allogeneic or syngeneic grafts.
Conclusions: This study demonstrates reduced preproEGF and preserved TGF-β_1 gene expression in chronically rejecting small bowel transplants.

Introduction

Chronic rejection is the predominant cause of graft dysfunction and failure of solid organ transplants, such as heart, kidney, liver and lung, which survive the first year post-transplantation.[1-3] The introduction of new potent immunosuppressive drugs to treat acute rejection has improved the survival of small bowel transplants. Recent clinical evidence demonstrates that the process of chronic rejection also hampers the long term survival of small bowel transplants, eventually leading to graft loss.[4-6] Understanding of the mechanisms of injury in this pathology is thus imperative for new therapeutic strategies to be developed. Chronic rejection of small bowel is characterized clinically by refractory diarrhea and weight loss and pathologically by mesenteric inflammation with sclerosis, myointimal proliferation of mesenteric vessels, hypertrophy of muscularis, scattered necrosis of crypt cells, crypt hyperplasia, blunting of villi and loss of goblet cells.[7-9]

Epidermal growth factor (EGF) and transforming growth factor-β_1 (TGF-β_1) have important, opposing, regulatory roles in normal intestinal physiology and alterations in their expression have been implicated in the pathophysiology of chronic rejection of other organs.[10-12]

EGF, a 53 amino acid peptide, stimulates the proliferation and differentiation of epidermal and epithelial cells and is mitogenic for other cell types such as fibroblasts and smooth muscle cells in vitro.[13,14] It regulates absorption of electrolytes and nutrients from the small intestine and is thus involved in the control of intestinal transport.[15] In the intestine EGF is produced by crypt cells and the enterocytes of the lower half of the villi.[16,17] Immunohistochemical studies have demonstrated EGF peptide in Brunner's glands of the duodenum and Paneth cells of the small intestine of adult rats.[18] EGF is formed as a large prepro molecule of 1217 amino acid residues[19] and processed to EGF 1-53.[20] Receptors for EGF are present throughout the entire gastrointestinal tract and have been localized to the brush border and basolateral membrane.[21-23]

In contrast to EGF, TGF-β_1 inhibits the proliferation of crypt cells and stimulates their differentiation to mature enterocytes.[24,25] TGF-β_1 also has important effects on extracellular matrix deposition, stimulating its synthesis and preventing its degradation, induces angiogenesis and plays a role in immunomodulation. These features make TGF-β_1 one of the main factors involved in the development of fibrosis in disease states.[26]

TGF-β in the small intestine is predominantly localized to the differentiated, non-proliferating cells of the villus tips.[25-27] Expression of TGF-β is regulated by both pre- and posttranscriptional mechanisms which include viral transactivators and growth factors. It is synthesized as a 391 amino acid residue precursor that is proteolytically cleaved to a 112 amino acid residue subunit and protein fragments which form a latency-associated peptide. It is secreted in a latent form, and conversion to the active form occurs at the cell surface and in the extracellular matrix.[28,29]

In the small intestine, TGF-β receptor mRNA assessed by Northern blot analysis was mainly shown in the non-epithelial compartment. Immunohistochemical localization demonstrated the TGF-β receptor was mainly expressed in the lamina propria and muscularis and to a lesser degree in the epithelium, where it was most prominent in the villus and absent in crypt cell.[30]

The aim of the present study was to evaluate the possible involvement of EGF and TGF-β_1 in the pathophysiology of chronically rejecting small bowel transplants.

Materials and methods

Groups

All experimental procedures involving animals conformed to the National Health and Medical Research Council Code of Practice and were approved by Monash Medical Centre Animal Ethics Committee B and the Monash Standing Committee on Ethics in Animal Experimentation.

Chronic rejection model

The model of chronic rejection after small bowel transplantation has been previously described.[9] In brief, male, adult inbred Dark-Agouti (DA) and Albino-Surgery (AS) rats were used, obtained from Monash Animal Services. One-step orthotopic total small bowel transplantation was performed, as described previously,[31] in the allogeneic DA to AS rat combination. Cyclosporin, 5mg/kg/day from day -2 until day 9, was administered to prevent acute rejection. Rats were killed at 50 days (n = 6) or 100 days post-transplantation (n = 5). Small bowel grafts were removed and processed for histopathology and Northern blot analysis.

Isografts
DA isografts were included to control for non-allogeneic injury. Rats were killed and grafts were removed at 50 (n = 6) or 100 days (n = 5) post-transplantation.

Normal rat small bowel(DA)
Four small bowels from normal DA rats were used as controls.

Intestinal Function
The function of the intestinal transplants at 100 days post-transplantation was investigated using the lactulose mannitol urinary excretion test. Animals were fasted during the day. 400 mg/kg D-lactulose and 100 mg/kg of D-mannitol were dissolved in 1 ml of water. This solution was administered orally by gavage. Each animal was placed in a metabolic cage and urine was collected overnight. The amount of urine produced was recorded, and samples were stored at -20 °C until analysis. Both lactulose and mannitol concentrations were measured colorimetrically on a Cobias bio analyzer and results are expressed as the lactulose: mannitol ratio.

Processing of the graft
Small bowel grafts were retrieved under ether anesthesia, and rats were killed immediately thereafter. Portions of the ileum measuring approximately 1 cm were fixed in 10 % buffered formalin and embedded in paraffin for histopathological studies or snap frozen in liquid nitrogen for RNA extraction.

Histopathology
Paraffin sections, stained with haematoxylin and eosin, were assessed in a blinded manner by two observers. The incidence of the following features were recorded for each of 9 high power fields (400 X): a) blunting of villi and goblet cell loss; b) crypt hyperplasia and necrosis; c) thickening of the muscularis; d) inflammatory cell infiltrate, vascular damage and sclerosis of the mesentery. Scores of 0, 1, 2 or 3 were assigned to features not observed, or observed in 1/3, 2/3, or all fields respectively. Results obtained for each feature were added up, and added scores of 0 were classified as no damage, 1-3 as very mild (grade 1), 4-6 mild (grade 2), 7-9 moderate (grade 3) and 10-12 severe (grade 4).

Northern blot analysis

Total RNA was extracted from 100 mg of snap frozen, full-thickness ileum using the RNAzol[tmB] method (Tel-test, Friendswood, USA). Northern blot analysis was performed as previously described.[10] Briefly, the RNA was denaturated with dimethylsulfoxide/glyoxal, electrophoresed in a 1 % agarose gel in phosphate buffer (pH 7.4) and blotted onto nylon membrane (Hybond-N, Amersham). Hybridization was carried out with a 400 bp mouse preproEGF cDNA probe[32] or a 370 bp TGF-β_1 cDNA probe[33], labeled with 32P-dCTP using random primers (Megaprime, Amersham). Quantitative variability of either isolation, transfer or loading of RNA was controlled for by reprobing the blots with a 720 bp cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).[10] Blots were prehybridized at 42°C. Hybridization with EGF and GAPDH probes was performed at 42°C and with TGF-β_1 cDNA probe at 55°C. Blots were exposed for 7 to 72 hours to a phosphor imager plate, and the bands of preproEGF, TGF-β_1 and GAPDH mRNA were measured using a Fuji Bio Imager Analyzer. Results are expressed as EGF/GAPDH or TGF-β_1/GAPDH ratios. Four normal DA ileum samples were run on every blot to enable comparisons between blots, and results obtained with normal DA were standardized to represent the value of 1.

Statistical analysis

For statistical analysis, the Kruskal-Wallis one way ANOVA followed by Mann Whitney U test and the Spearman correlation test were used. The results are expressed as mean ± SEM; probability of $p < 0.05$ was accepted as significant.

Results

Histology

The evolution of histological changes in this model of chronic rejection has been described previously.[9] All allogeneic transplants developed chronic rejection although the severity of damage varied between animals from very mild to moderate. At 50 days, 4 out of 6 animals had moderate chronic rejection. A progression of chronic damage with time was not evident. At one hundred days post-transplantation, 2 of 5 rats had moderate changes. All other grafts had mild changes. None or very mild (1 out of 11 animals) changes were seen in the syngeneic grafts.

Intestinal Function

The lactulose-mannitol urinary excretion ratio was significantly elevated in allografts compared to isografts at 100 days (Table 1), suggesting an impaired mucosal integrity in chronically rejecting intestinal transplants. There was no significant difference between the syngeneically transplanted group and control rats. ($p = 0.89$)

	Normal DA	n	DA-DA	n	DA-AS	n
Lactulose/ Mannitol ratio	0.37 ± 0.02	(4)	0.54 ± 0.17	(4)	1.34 ± 0.15 *	(5)

Table 1. Lactulose-mannitol excretion ratio in small bowel transplants
At 100 days post-transplantation, intestinal function was measured by the urinary excretion of orally administered lactulose and mannitol. The ratio lactulose / mannitol was calculated and results are expressed as mean ± SEM. * = $p < 0.05$ vs DA-DA and normal DA.

Northern blot analysis of TGF-β_1 mRNA

The results of Northern blot analysis of TGF-β_1 expression are summarized in Table 2. No significant changes in TGF-β_1 expression were detected in either the allogeneic or syngeneic grafts at the time points examined.

TGF-β_1/GAPDH	Normal DA	n	DA-DA	n	DA-AS	n
day 50	1.00 ± 0.08	(4)	1.35 ± 0.09	(6)	1.17 ± 0.16	(7)
day 100	1.00 ± 0.09	(4)	1.02 ± 0.16	(5)	0.83 ± 0.09	(5)

Table 2. TGF-β1 gene expression in small bowel transplants.
Ileum RNA samples were isolated and Northern hybridization was performed with preproEGF and GAPDH cDNA probes as described in the Methods. Results are expressed as TGF-β_1/GAPDH ratios, standardized to normal DA. Units are arbitrary. Data were analyzed by Kruskall Wallis nonparametric ANOVA, and pairs were compared by Mann-Whitney U test.

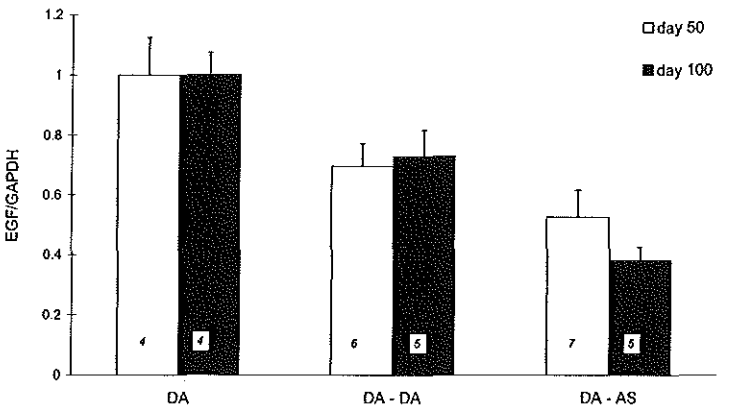

Figure 1. Prepro EGF mRNA in small bowel transplants at 50 and 100 days post-operatively. Ileum RNA samples were isolated and Northern hybridization was performed with preproEGF and GAPDH cDNA probes as described in the Methods. Results are expressed as EGF/GAPDH ratios, standardized to normal DA. Units are arbitrary. Number of animals are shown inside the columns. * = $p < 0.05$ vs DA-DA (Kruskal Wallis one way ANOVA followed by Mann Whitney U test).

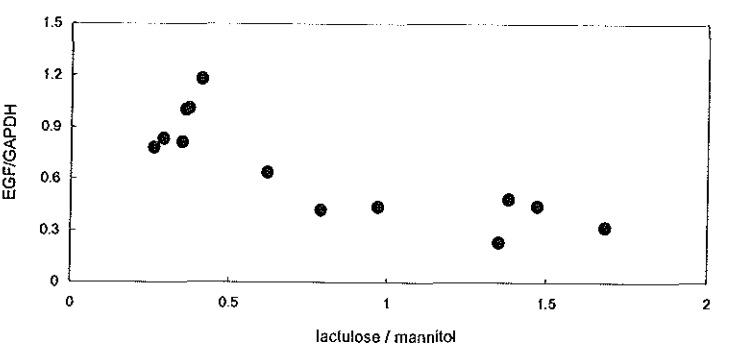

Figure 2. Relation between EGF gene expression and intestinal function.
Ileum RNA samples were isolated and Northern hybridization was performed with preproEGF and GAPDH cDNA probes as described in the Methods. Results are expressed as EGF/GAPDH ratios, standardized to normal DA. At 100 days post-transplantation, the intestinal function was measured by the urinary excretion of lactulose and mannitol after oral administration and expressed as a ratio lactulose / mannitol. Data were analyzed with the Spearman correlation test: $R = -0.74$, $p = 0.01$, $n = 14$.

Northern blot analysis of prepro EGF mRNA

The results of Northern blot analysis of prepro EGF mRNA are depicted in Figure 1. Allografts demonstrated a progressive reduction in preproEGF mRNA levels over time, which attained significance at 100 days post-transplantation when compared to their relevant control, DA isografts ($p = 0.03$). The preproEGF expression of DA isografts did not significantly differ from untransplanted, normal DA controls ($p = 0.06$).

The EGF expression was inversely correlated with the lactulose-mannitol excretion (-0.74, $p = 0.01$, $n = 14$)(Figure 2).

Discussion

Chronic rejection hampers long term survival of organ transplants, including small bowel grafts.[4-6,34] To investigate the pathophysiology of chronic rejection after small bowel transplantation, we recently developed a rat model in which allogeneic small bowel grafts demonstrated histological features of chronic rejection.[9] The present study investigates the expression of EGF and TGF-β_1 in the model of chronically rejecting small bowel transplants.

PreproEGF gene expression was significantly reduced in chronically rejecting small bowel allotransplants 100 days post-transplantation compared to syngeneic controls. The reduced preproEGF expression could be the result of loss of EGF producing cells through chronic graft injury, or could be the response to downregulatory signals. Ischaemic injury may be an important factor resulting in the loss of EGF producing cells in the intestinal crypts. Since the mesenteric vessels of the allografts were found to be partially obliterated in this model, the resulting ischaemia may have contributed to the scattered necrosis of crypt cells and crypt hyperplasia seen in chronic rejection of small bowel allografts. This is consistent with the sequence of events described by Langrehr *et al.* in another model of chronic intestinal rejection in which the early stages were characterized by inflammation and endothelialitis of the mesenteric vessels, while mucosal inflammation, apoptosis and cryptitis emerged with time.[8] Furthermore, ischaemia has been identified as a signal resulting in down-regulation of preproEGF mRNA while the overall RNA transcription is not affected.[35] Other factors which may have provided downregulatory signals include inflammatory mediators such as interleukin-1 and TGF-β, which have

been shown to modulate growth factor expression.[36,37]

Since EGF stimulates the proliferation of crypt cells[24] and thus functions to maintain mucosal integrity, blunting of villi and loss of goblet cells could be the result of or be aggravated by the reduced preproEGF production. In addition, the impaired mucosal integrity, as measured by the lactulose-mannitol test, could also directly reflect to decreased EGF production: EGF has a cytoprotective effect through stimulation of goblet cells to release mucus.[38]

Administered EGF has been shown to heal and prevent experimentally induced gastrointestinal ulcerations in animals.[39-41] In a rat model of acute abdominal radiation EGF administration decreased the mucosal ulceration, infiltration of polymorphonuclear lymphocytes and maintained the cellular structure.[42] Guglietta & Sullivan have described possible clinical applications of EGF[43] and based on the results of our study, administration of exogenous EGF emerges as a possible treatment strategy to ameliorate the process of chronic rejection.

TGF-β_1 gene expression was not modified in chronically rejecting small bowel allografts compared to isografts or normal bowel. This finding was unexpected since in other models of chronic organ rejection, using the same rat strain combinations, an increase of the TGF-β_1 mRNA transcripts was found.[33] Using a different strain combination, TGF-β_1 was increased in a model of chronic small bowel rejection.[44] A possible explanation for this discrepancy might be a difference in mucosal damage: In our model loss of goblet cells was a consistent feature of chronic rejection while this was not described in Walgenbachs model. Since the allografts demonstrated a significant increase of ED-1 positive macrophages/ monocytes [9] which are known to be potential producers of TGF-β_1, an increased TGF-β_1 expression by the macrophages and a concomitant decreased production of TGF-β_1 mRNA by injured enterocytes could explain our finding.

To investigate the possible role of alloantigen-independent factors such as ischemia/reperfusion and surgical trauma on the process of chronic rejection, DA-DA isografts were compared to native DA bowel. Isografts preserved normal histology, normal function and their expression of EGF and TGF-β_1 was not significantly different from that of the normal DA controls, although the EGF expression in isografts showed a tendency to be lower. Similarly, Sigalet *et al.* found that intestinal transplantation per se had only minimal effects on intestinal function.[45] Thus, in contrast to the reported importance of alloantigen-independent factors in kidney transplantation,[46] these factors do not seem to play a major role in our small bowel transplantation model at the timepoints studied.

In summary, this study demonstrates reduced preproEGF and preserved TGF-β_1 gene expression in chronically rejecting small bowel transplants. A possible beneficial effect of exogenous administration of EGF to the long-term survival of small bowel allografts is currently under investigation.

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Increased expression of basic fibroblast growth factor during chronic rejection in intestinal transplant is associated with macrophage infiltrate

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Summary

Background: Long-term survival of intestinal transplants is hampered by chronic rejection (CR). Since transplants with CR demonstrate fibrotic changes, the cytokine basic fibroblast growth factor (bFGF) could be involved in the tissue remodelling of chronic intestinal rejection.
Aims: To investigate the bFGF gene and protein expression and distribution in chronically rejecting intestinal allografts.
Methods: Orthotopic small bowel transplantation was performed in the allogeneic DA to AS rat combination; Cyclosporin was administered temporarily to prevent acute rejection. Controls were DA isografts and normal DA. bFGF gene expression was evaluated using RT-PCR of the ileum-RNA and standardised against GAPDH expression. bFGF protein was demonstrated using immunohistochemistry. To identify the bFGF-positive cell type, stains for cell markers were performed on sequential sections.
Results: Allografts showed histological features of CR, whereas isografts preserved normal architecture. bFGF gene expression was present in normal ileum and significantly upregulated in allografts. Immunohistochemical staining showed a significant increase of bFGF protein compared to isografts. Most $bFGF^+$ cells were localised in the submucosa and muscularis, particularly around neural plexus. $bFGF^+$ cells appeared to be $ED\text{-}2^+$ macrophages, strongly suggesting that the site of bFGF production is the activated macrophage.
Conclusions: This study demonstrates increased bFGF mRNA and protein in chronically rejecting intestinal allografts, which appeared to be produced by macrophages.

Introduction

With the improved treatment of acute rejection episodes, intestinal transplantation has evolved over the last decade from a cumbersome procedure with unacceptably high morbidity and mortality to a more acceptable therapy for those with permanent intestinal failure. Although long-term outcome is currently mainly determined by uncontrolled acute rejections and lethal infections, it is also hampered by chronic rejection,[1,2] which is the leading cause of late graft loss of other organs such as heart and kidney.[3,4] Indeed, some patients have already lost their small bowel transplant due to chronic rejection.[5,6] In contrast to the knowledge of the nature of the immune response and production of cytokines during acute intestinal rejection,[7] little is known about the involvement of cytokines in the pathophysiology of chronic rejection of intestinal transplants: The Pittsburgh-group has given some attention to this issue. They demonstrated an increased expression of the cytokines IL-4 and interferon-γ in chronically rejecting intestinal allografts, mainly localised in the thickened muscularis externa.[8,9] To further understand the mechanisms of tissue remodelling during chronic rejection of the small bowel, we have recently developed a rat model.[10] Histomorphological analysis demonstrated mesenteric inflammation with sclerosis, myointimal proliferation of mesenteric vessels, thickening of the muscularis, crypt hyperplasia, blunting of villi and loss of goblet cells, features which have also been found both in humans and other animal models.[2,11,12] Some of these characteristics are consistent with the possible involvement of fibrogenic growth factors such as basic fibroblast growth factor (bFGF), as demonstrated for other organs.[13,14]

bFGF, or fibroblast growth factor-2 (FGF-2), a member of the FGF family, is a 133 to 154 amino acid (17.5 kDa) peptide which is mitogenic for vascular endothelial cells, smooth muscle cells, fibroblasts, neural cells and chemotactic for macrophages and endothelial cells. It has been involved in wound healing and tissue regeneration.[15] bFGF has a high affinity for heparin and heparan sulphate proteoglycans, and can thus be found in the extracellular matrix, or bound to basement membranes.[16] The binding of bFGF to heparan sulphate proteoglycans, protects it from inactivation by endogenous proteolytic agents.

In the digestive tract, bFGF peptide has been detected in duodenum,[17] and in normal gastric and colonic mucosa. It has been immunohistochemically localised to vessels, fibroblasts and macrophages, with epithelial cells and

extracellular matrix demonstrating no bFGF immunoreactivity.[18] Heparan sulphate proteoglycans act as the low-affinity receptors for bFGF in the intestine,[19] forming an FGF-heparin complex, which facilitates the interaction between FGFs and their high-affinity cell surface tyrosine kinase receptors, identified on various cell types including smooth muscle cells, endothelial cells, macrophages and intestinal epithelial cells.[20,21]

The function of bFGF in the small intestine is not well defined. bFGF failed to promote epithelial restitution after colonic mucosal damage in vitro.[22] Production of bFGF in the adult ileum has not yet been described. However, bFGF is known to be produced by activated macrophages, smooth muscle cells and fibroblasts,[15,20] and just these sources of bFGF could be important in the tissue remodelling processes of chronic intestinal rejection.

The aim of the present study was to evaluate the possible involvement of bFGF in the pathophysiology of chronic intestinal allograft rejection by analysing gene and protein expression and distribution in the course of chronic rejection.

Materials and methods

Groups

All experimental procedures involving animals conformed to the National Health and Medical Research Council Code of Practice and were approved by Monash Medical Centre Animal Ethics Committee B and the Monash Standing Committee on Ethics in Animal Experimentation.

Chronic rejection model

The model of chronic rejection after small bowel transplantation has been previously described.10 In brief, male, adult inbred Dark-Agouti (DA)(RT-1^{av1}) and Albino-Surgery (AS)(RT-1^{l}) rats were used, obtained from Monash Animal Services. One-step orthotopic total small bowel transplantation was performed, as described previously,23 in the allogeneic DA to AS rat combination. Cyclosporin, 5mg/kg/day from day -2 until day 9, was administered to prevent acute rejection. Rats were killed at 50 days (n = 6) or 100 days post-transplantation (n = 5). Small bowel grafts were removed and processed for histopathology, immunohistochemistry and reverse transcriptase polymerase chain reaction.

Isografts
Untreated DA isografts were included to control for non-allogeneic injury. Rats were killed and grafts were removed at 50 (n = 6) or 100 days (n = 5) post-transplantation.

Normal rat small bowel(DA)
Four small bowels from age-matched, normal DA rats were used as controls.

Processing of graft
Small bowel grafts were retrieved under ether anaesthesia, and rats were killed immediately thereafter. Portions of the ileum measuring approximately 1 cm were fixed in 10 % buffered formalin and embedded in paraffin for histopathological studies or snap frozen in OCT for immunohistochemistry or directly in liquid nitrogen for RNA extraction.

Histopathology
Paraffin sections, stained with haematoxylin and eosin, were assessed in a blinded manner by two observers. The incidence of the following features were recorded for each of 9 high power fields (400 X): a) blunting of villi and goblet cell loss; b) crypt hyperplasia and necrosis; c) thickening of the muscularis; d) inflammatory cell infiltrate, vascular damage and sclerosis of the mesentery. Scores of 0, 1, 2 or 3 were assigned to features not observed, or observed in 1/3, 2/3, or all fields respectively. Results obtained for each feature were added up, and added scores of 0 were classified as no damage, 1-3 as very mild (grade 1), 4-6 mild (grade 2), 7-9 moderate (grade 3) and 10-12 severe (grade 4).

Immunohistochemistry
Immunoreactive bFGF was assessed on 4 µm cryostat sections post fixed in PLP by a four-layer immunoperoxidase technique. Briefly, non-specific binding was blocked by preincubation with 10 % normal rabbit serum (Dako, Christchurch, New Zealand), in phosphate buffered saline (PBS) with 0.01% sodium azide at room temperature. This was followed by overnight incubation with the primary mouse anti-bFGF (Upstate Biotechnology Incorporated) and cell surface marker ED-2 (a gift by dr.C.D. Dijkstra, Amsterdam, The Netherlands). After each incubation, slides were washed in PBS-0.2% gelatine. A second layer, goat anti-mouse IgG (Sigma, Castle Hill, Australia) was then applied for 30 minutes.

Endogenous peroxidase activity was blocked by incubation for 10 minutes in methanol/0.3% H_2O_2, after dehydration through graded alcohol. After rehydration and washing, the third and fourth layer, rabbit-anti goat immunoglobulin (Dako) and goat peroxidase anti-peroxidase (Dako) were applied both for 30 minutes. The reaction was developed by the addition of metal enhanced Diaminobenzidine substrate (Pierce,) and slides were counterstained in Harris haematoxylin, dehydrated, cleared and mounted.

Negative controls included sections where the primary antibody was omitted for every animal. Isotype-specific control monoclonal antibodies (Dako) were also used. No staining was observed with these irrelevant antibodies. The specificity of the antibodies to bFGF was confirmed by a solid phase absorption study, as previously described.[24]

The immunohistochemical staining was analysed by two observers, 'blind' as to treatment group and time post-transplantation. Positive cells were counted in four different compartments of the ileum: villi, crypts, submucosa and muscularis, and are expressed as numbers of positive cells/ 0.1 mm^2.

Semiquantative Reverse Transcriptase Polymerase Chain Reaction

RNA preparation

Total RNA was extracted from approximately 100 mg of snap frozen full-thickness ileum using the RNAzol^{tmB} method (Tel-test, Friendswood, USA). RNA was quantified spectrophotometrically and samples were diluted to 2.5 µg/µl.

cDNA synthesis

Reverse transcription reactions were set up using 1 µl of RNA (2.5 µg), in 20 µl volumes using an MULV RT system (Perkin Elmer, Foster City, CA, USA)

PCR DNA amplification

PCR reactions were performed using a PCR amplification kit, with AmpliTaq gold (Perkin Elmer). The co-amplification reaction for bFGF and GAPDH genes was optimised to ensure that the amplification of both genes was within the exponential phase at the end-point of the PCR. Different concentrations of the primer pairs and of the Amplitaq gold were tested. Based on optimisation experiments, PCR amplification reactions were performed in 25 µl volumes and contained 1.5 mM $MgCl_2$, x 1 PCR buffer, 1.25 U AmpliTaq gold, 100 nM bFGF, 100 nM GAPDH and 2.5 µl cDNA. The following primers were used: bFGF sense: Fluorescein (Fl)-

TCACTTCGCTTCCCGCACTG (549), antisense CCAGCAGCCGTCCATCTT (787); GAPDH sense: Fl-CCTTCATTGACCTCAACTACATG (131), antisense: GATGACCTTGCCCACAGCCTT (667). The position of the 5 nucleotide of each primer in the published sequences[25,26] is included in parenthesis. Following an activating step for 12 minutes at 94 °C, 35 cycles were performed with a denaturation step of 1 minute at 94 °C, an annealing temperature for 1 minute and 30 seconds started at 72 °C and declining with 0.4 °C per cycle until 64°C, and an extension step of 2 minutes at 72 °C. The resulting PCR products had a length of 238 and 536 bp for bFGF and GAPDH respectively. The GAPDH primers were designed by Dr P.Aldred and kindly provided by Dr N. Cranswick. No interference was demonstrated between primer pairs.

Analysis of PCR products

The sense primers were labelled with fluorescein at the 5' end for direct detection of the PCR product bands using a laser based fluorescence DNA detection system (Fluorimager 575-Molecular Dynamics, Sunnyvale, CA, USA). The volume of the individual bands was integrated using the ImageQuant software (Molecular Dynamics) and ratios between bFGF and GAPDH bands were calculated. Four normal DA ileum samples were amplified and run with each group containing all isografts or allografts, at day 50 or day 100 to enable comparisons between each group of amplified samples, and results obtained with normal DA were standardised to 1. All samples were amplified and run in duplicate.

Statistical analysis

All statistical analysis were performed using SPSS for Windows. Kruskal-Wallis nonparametric ANOVA followed by Mann Whitney U test and Spearman correlations were used. The results are expressed as mean ± SEM; probability of $p < 0.05$ was accepted as significant.

Results

Histology

All allogeneic transplants developed CR although the severity of damage varied between animals from very mild to moderate. At 50 days, 4 out of 6 animals had moderate chronic rejection (grade 3). A progression of chronic damage with time

was not evident. At one hundred days post-transplantation, 2 of 5 rats had moderate changes. All other grafts had mild changes. Syngeneic grafts retained a normal architecture.

Immunohistology

Normal ileum (Figure 1a, Figure 2)
The highest density of bFGF protein expressing cells was located in the submucosa; a few positive cells were detected in the lamina propria of the villi and crypts, whereas epithelial cells were negative. bFGF positive cells were present throughout the muscularis, their density being highest between the circular and longitudinal layers. Vessels did not show bFGF reactivity.

Intestinal isografts
The number and distribution of bFGF protein expressing cells did not differ from normal controls, both at day 50 and day 100 (Figure 2).

Intestinal allografts (Figure 1c, Figure 2)
The number of bFGF protein expressing cells in allografts was approximately doubled compared to isografts and normal DA.($p < 0.05$ and $p < 0.01$, respectively) at 50 and 100 days. Increased numbers of bFGF positive cells were seen in all 4 compartments evaluated. As in normal ileum, most bFGF positive cells were localised to the submucosa. Some positive cells were detected in the lamina propria of the villi and crypts, whereas no bFGF positivity was seen in epithelial cells. As in the controls, the highest number of cells was found between the circular and longitudinal muscular layers. None bFGF positivity was seen in the vessels.

We had previously demonstrated increased numbers of monocytes /macrophages (ED1-positive cells) and T cells in chronic rejection of small bowel.[10] Staining of sequential sections was performed to identify the lineage of the bFGF positive cells in our model.

As shown in Figures 1b and 1 d, many cells expressing bFGF protein appeared in sequential sections to be ED-2 positive tissue macrophages. Immunohistochemical staining of normal DA ileum with ED-2 antibody demonstrated that the highest number of ED-2 positive cells was present in the submucosa. Very few tissue macrophages were present in the villi. The density was somewhat higher in the crypts. There were some positive cells in the muscularis, with accumulation at

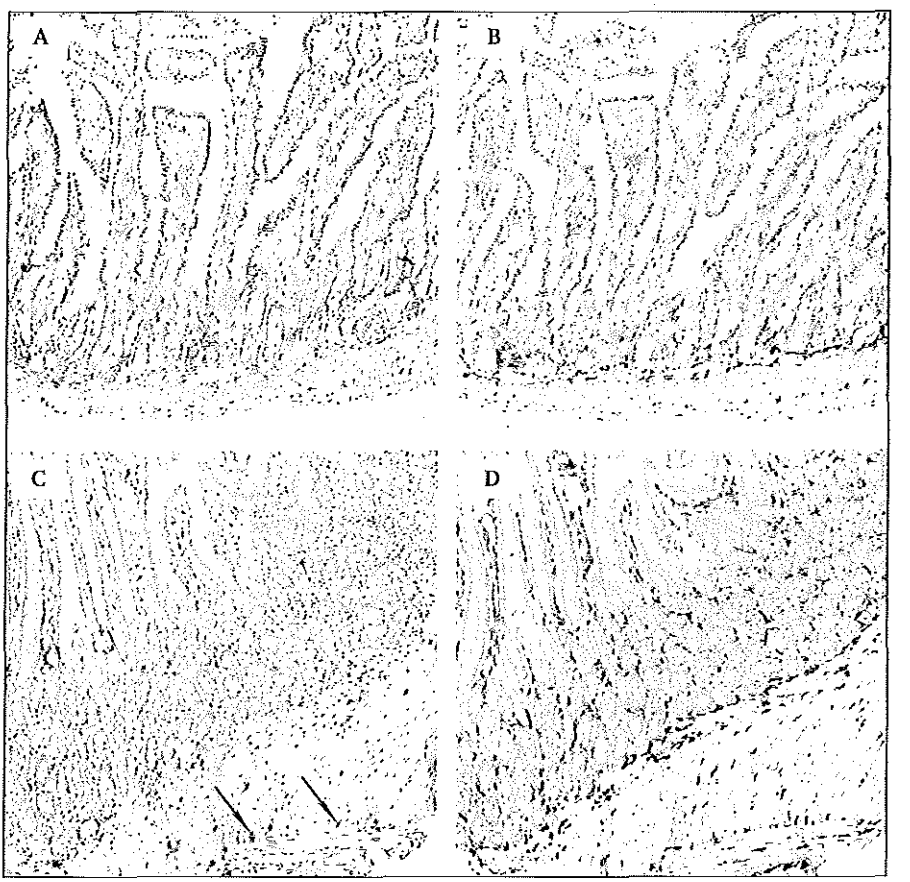

Figure 1. a) bFGF protein expression in a normal small intestine b) normal distribution of tissue macrophages in small intestine of the DA-rat. c-d)Chronically rejecting intestinal allografts demonstrating an increased number of bFGF positive cells and tissue macrophages. The highest increase was found in the submucosa and in the muscularis, particularly between the circular and longitudinal layers (arrow).

the border between the circular and longitudinal layer. Isografts did show a progressive increase of tissue macrophages over time, which attained significance at 100 days post-transplantation when compared to normal controls ($p < 0.05$) (Figure 2). This increase was found in all compartments, although it did not reach statistical significance in the crypts. Fifty days after transplantation, allografts demonstrated a significant increase in ED-2 positive macrophages compared to isografts and to normal DA ileum ($p < 0.01$). Increased macrophage numbers were evident in villi, crypts, submucosa and muscularis, with the highest density found in the submucosa. Hundred days after engraftment, the number of ED-2 positive macrophages in allografts was still significantly increased compared to isografts and normal ileum ($p < 0.01$ and $p < 0.02$, respectively), but did not differ significantly from day 50. The same pattern of distribution was observed.

The association between bFGF expression and tissue macrophages was further examined by performing a correlation study between bFGF protein expressing cells and ED-2 positive macrophages. Taking all compartments together, bFGF protein expression was found to be significantly correlated with the number of tissue macrophages ($R = 0.86$, $p < 0.001$, $n = 25$). (Figure 3). Furthermore, the number of bFGF protein expressing cells was significantly correlated with the severity of chronic rejection ($R = 0.80$, $p < 0.001$, $n = 21$) (Figure 4).

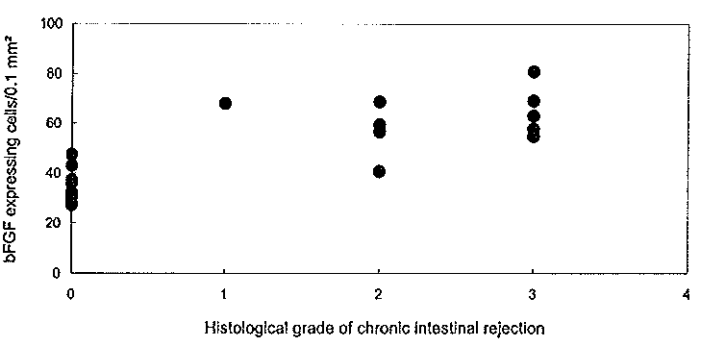

Figure 4. bFGF protein and the histological grade of chronic intestinal rejection. The severity of chronic changes including blunting of villi and goblet cell loss, crypt hyperplasia and necrosis, thickening of the muscularis, inflammatory cell infiltrate vascular damage and sclerosis of the mesentery were classified as no damage (grade 0), very mild (grade 1), mild (grade 2), moderate (grade 3) or severe damage (grade 4). The number of bFGF protein expressing cells were counted and given as a quantity per 0.1 mm2. Data were analysed by Spearman correlation: $R = 0.80$, $p < 0.001$,$n= 21$

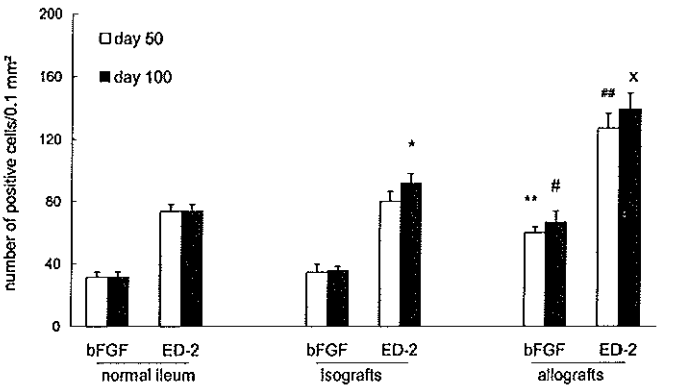

Figure 2. bFGF protein expression and ED-2 positive tissue macrophages in the course of chronic intestinal rejection. Grafts were processed for immunohistology and stained for immunoreactive bFGF protein and ED-2 tissue macrophages as described in the Methods. Results are expressed as the number of bFGF protein expressing cells and ED-2 positive cells and mean ± SEM are given. * = $p < 0.05$ vs normal ileum; ** = $p < 0.01$ and $p < 0.05$ vs normal ileum and isografts, respectively: # = $p < 0.05$ and $p < 0.02$ vs normal ileum and isografts, respectively; ## = $p < 0.01$ vs normal ileum and isografts; x = $p < 0.02$ vs normal ileum and isografts.

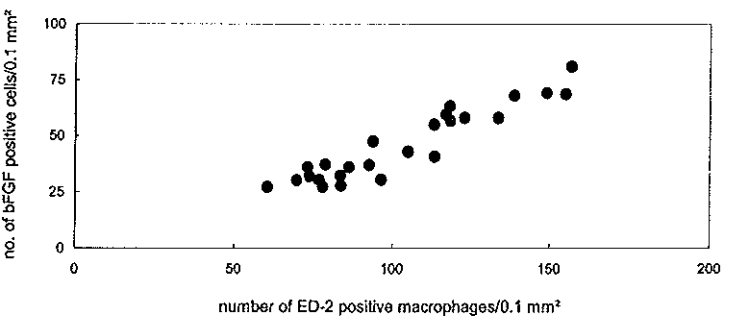

Figure 3. Correlation between the number of bFGF expressing cells and the number of $ED2^+$ tissue macrophages in normal ileum and intestinal transplants at timepoints studied. Grafts were processed for immunohistology and stained for immunoreactive bFGF protein and ED-2 tissue macrophages. The number of bFGF protein expressing cells and $ED\text{-}2^+$ cells were counted and given as a quantity per 0.1 mm^2 Data were analysed by Spearman correlation: $R = 0.86$, $p < 0.001$, $n = 25$.

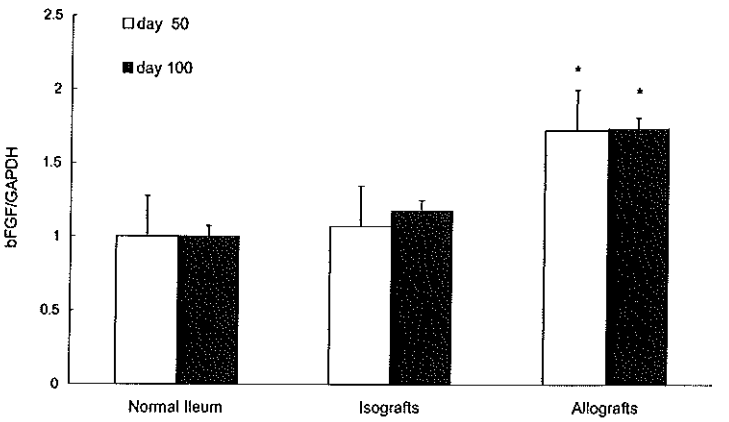

Figure 5. bFGF gene expression in chronically rejecting intestinal allografts
Ileum RNA samples were isolated and RT-PCR was performed with bFGF and GAPDH primers as described in the Methods. Results are expressed as bFGF/GAPDH ratio, standardised to normal DA ileum and mean ± SEM are given. Units are arbitrary. * = $p < 0.02$ vs normal DA ileum (Kruskal Wallis one way ANOVA followed by Mann Whitney U test)

RT-PCR

The bFGF gene was transcriptionally active in normal DA ileum (Figure 5). Allografts demonstrated a significant increase in bFGF mRNA levels at 50 and 100 days post-transplantation, compared to normal controls ($p < 0.01$ and $p < 0.02$ respectively). The bFGF mRNA of isografts was not postoperatively modified compared to normal DA controls. bFGF gene expression was correlated with the histological grade of chronic rejection ($R = 0.55$, $p < 0.02$, $n = 21$)

Discussion

Long-term survival of intestinal transplants is hampered by chronic rejection, the major cause of graft loss of other transplanted organs, such as kidney and heart.[3,4] Chronic rejection of transplanted organs is characterised by a inflammatory infiltrate, graft arteriosclerosis and fibrosis. Small bowel-specific features include thickening of the muscularis externa, crypthyperplasia, and blunting of villi.[2,10,12] Because of its effects on smooth muscle cells, fibroblasts and the extracellular matrix, bFGF is a candidate for being involved in the tissue

remodelling of chronic rejection in intestinal transplants, as demonstrated in other organs.[13,14] Moreover, bFGF has been identified to act as a chemoattractant for macrophages,[27] a cell type which has been recurrently demonstrated in chronically rejected organs.

The present study demonstrates that the bFGF gene was transcriptionally active in the ileum. This is the first time that bFGF mRNA has been detected in the adult intestine. Chowdhury *et al.* could not detect any signal in normal colorectal mucosa using RT-PCR.[28] Shimasaki *et al.* found that bFGF measured by Northern blot analysis could not be detected in the small intestine.[25] Our use of the full thickness of the intestine combined with a sensitive RT-PCR protocol may explain these differences. The bFGF protein was also present in normal and rejecting ileum.

The main finding of this study is that there was a significant increase in bFGF mRNA and protein in chronic intestinal allograft rejection. This enhanced bFGF expression appeared to be correlated with the histological damage of the intestinal grafts, further suggesting a role for this growth factor in the tissue remodelling. In contrast, chronic vascular rejection in lung allografts did not coincide with bFGF immunoreactivity; bFGF was only markedly upregulated in the early phase and restricted to peribronchiolar and perivascular infiltrates.[14] Also, bFGF did not correlate with the degree of transplant vasculopathy in bioptied heart allografts [29,30] and in aorta allografts.[31] Indeed, myoproliferation of the intra-intestinal arteries was also not accompanied by enhanced bFGF immunoreactivity. Therefore, bFGF appears to be important in organ-specific remodelling, i.e. muscular thickening and neuron repair, rather than being involved in transplant arteriosclerosis.

Many of the bFGF expressing cells appeared to be ED-2 positive macrophages. Furthermore, the number of bFGF protein and ED-2 positive cells were significantly correlated, strongly suggesting that the macrophage is an important source of bFGF. Lee *et al.* also found that bFGF protein was mainly expressed by infiltrating cells, including macrophages, in lung allografts.[14] The production of bFGF by macrophages has been unequivocally demonstrated by PCR and Northern blot analysis of macrophage-mRNA.[20]

bFGF expressing cells and tissue macrophages in allografts were mainly localised to the submucosa and the muscularis, particularly between the longitudinal and circular layer. Since the neural plexus are localised in these areas, bFGF and macrophages have not only to be associated to the muscular

thickening, but also might act in the intrinsic neural plexus. Lee *et al.* found a suppressed neuromuscular transmission during subclinical chronic intestinal rejection, which is related to the loss of enteric neurons as a consequence of extrinsic denervation.[32] Since bFGF is known to stimulate neurons outgrowth,[15] the preferential site of bFGF expressing cells could reflect an attempt to repair the intrinsic neural plexus. We could not detect bFGF protein immunohistochemically in epithelial cells of normal intestine, consistent with the results of Ohtani *et al.*[18] Moreover, no staining was seen in the damaged mucosa of chronically rejecting intestinal allografts, suggesting that endogenous bFGF is not involved in the repair of epithelial damage in CR.

Although the influx of macrophages in isografts was significantly increased when compared to normal ileum, histologically the intestinal grafts were well preserved during follow-up. Also, bFGF protein and mRNA remained at non-transplanted level, further assuming that the macrophages in the isografts were inactive.

Based on the present data, we postulate that bFGF may have important effects which contribute to chronic rejection of intestinal grafts: bFGF is a chemoattractant for macrophages, contributing to the persistent increase of macrophages in chronic intestinal rejection. Activated macrophages in turn produce bFGF and other fibrogenic cytokines, including TGF-beta and PDGF and have been shown to have a pivotal role in the process of chronic rejection.[33,34] bFGF also has a mitogenic effect on smooth muscle cells, enhancing the hypertrophy of the muscularis. The localisation of bFGF also suggests that it may have a positive effect on neuron outgrowth.

To further understand the contribution of bFGF in chronic rejection and its relationship with tissue macrophages, studies on the effect of neutralisation of bFGF protein and on the effect of a selective inhibition of macrophages should be performed.

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II

Summary and General Discussion

Summary

Chronic transplant dysfunction (CTD) is the predominant cause of late graft failure. The common histopathological feature in all transplanted organs is intimal hyperplasia, accompanied by organ-specific lesions. The knowledge about the pathogenesis of CTD is incomplete. There is still no treatment to prevent CTD and retransplantation is currently the only effective therapy.

The studies described in this thesis were performed to investigate etiological and pathophysiological aspects of CTD, with the ultimate goal to obtain new therapeutic strategies.

Clinical and experimental studies suggest that non-alloantigen specific factors play a role in the process of CTD. Since a role for ischemia in the process is still controversial, our purpose was to clarify its contribution to CTD. At first, we studied the effect of prolonged cold ischemia on the development of the cardinal feature of CTD, i.e. intimal hyperplasia, in an isolated fashion. Therefore, we transplanted aortic grafts in a high-responder allogeneic (WAG-BN) rat strain combination. Cold preservation (4°C) of the aortic grafts in UW-solution for 24 hr did not enhance the degree of intimal hyperplasia in this non-immunosuppressed allogeneic model. Likewise, in BN-BN aortic isografts that show minimal intimal hyperplasia at week 4 after transplantation, 24 hours preservation did not adversely influence its development (**Chapter 2**).

The question remained whether cold ischemia affects the parenchyma of a graft. In addition, the susceptibility of endothelium to ischemic injury might be site-dependent. To mimic clinical organ transplantation, the influence of immunosuppression on ischemia also had to be studied. From a clinical point of view, the effect of cold ischemia is most relevant in a kidney transplantation model; kidney transplants are preserved from 2 hours in living-related programs to up to 40 hours using cadaveric donors. In syngeneic (BN-BN) kidney transplants, the transplant procedure itself, which includes an ischemic time of 30 minutes, did not affect function and morphology during a 1-year observation period (**Chapter 3**).

Kidney isografts subjected to 24 hr cold ischemia, however, developed progressive proteinuria from week 18 onwards, and showed 1 year after transplantation histological features of CTD: intimal hyperplasia, glomerulopathy, and tubular atrophy. In the first days after transplantation, there was an increased infiltration of $CD4^+$ T-cells and macrophages, which coincided

with upregulated ICAM-1 expression on the endothelium. At 1 year, the numbers of these infiltrating cells were still significantly increased compared to those in isografts with a minimal ischemic period. Exposure of the donor kidney to 24 hr of cold ischemia in allografts (BN-WAG) did not affect the onset and progression of CTD. Ischemic and non-ischemic allografts, immunosuppressed with Cyclosporine for 3 weeks developed a similar pattern of CTD in 26 weeks. As in ischemic isografts, infiltrating cells mainly consisted of $CD4^+$ T cells and macrophages, albeit that their numbers were higher, in particular in the early phase. This discrepancy of the effect of prolonged cold ischemia between iso- and allografts appeared to be related to Cyclosporine. Administration of the same immunosuppressive regimen to ischemic isografts led to inhibition of cold ischemia induced late renal dysfunction (**Chapter 4**).

Another indication that Cyclosporine inhibited ischemic mediated injury in long-term allografts comes from observations, described in **Chapter 5**. In the absence of Cyclosporine ischemia augmented the alloimmune mediated response in the BN-WAG allografts: Interstitial infiltrates, consisting mainly of $CD4^+$ T cells, and tubulitis occurred earlier in the grafts subjected to 24 hr cold ischemia.

Experiments described in the **Chapters 2** and **6** show that surgery-related factors also have an impact on the development of CTD. For example, in the syngeneic aorta transplantation model, the mode of suturing influences the development of intimal hyperplasia. In the midportion of the 1 cm aortic transplant, continuous suturing of the anastomosis led to more intimal hyperplasia than interrupted suturing. Aortic stretch – performed by transplanting 8 mm long aorta segments into 12 mm dissected recipients – did not influence the degree of intimal hyperplasia, but did decrease medial thickness. In the syngeneic kidney transplantation model, we demonstrated that an uretero-neocystostomy as surgical technique to restore the urinary tract contributes to long-term functional and histological renal changes. As the intrapelvic pressure was significantly increased, a partial obstruction appears to be cause of the renal impairment.

Since some transplant studies suggest differences in graft outcome due to ethnicity of the donor organs, intrinsic genetic factors of an organ may contribute to CTD. **Chapter 7** presents the effects of transplanting donor kidneys of different rat strains into a histocompatible F_1-recipient, allowing these grafts to be exposed to the same blood pressure profiles, metabolic and hormonal environment. The ACI strain, resistant to hypertension induced renal damage, and the hypertensive

FHH rat, susceptible for hypertension-induced renal injury, and the (ACI x FHH) F_1 were used as donors. In the normotensive F_1 recipient with a F_1 or ACI transplant, function and morphology did not differ from their unilaterally nephrectomized controls. In contrast, the FHH kidney showed a progressive deterioration of function and histological features of CTD. Genetic susceptibility of a donor kidney appears to be an important factor in the etiology of CTD.

Chapter 8 describes that small bowel allografts developed functional abnormalities and histological signs of CTD by 50 and 100 days after transplantation. $CD4^+$ -, $CD8^+$ T cells and macrophages infiltrated the grafts. B-lymphocytes and NK-cells did not appear to be involved. Cyclosporine treatment delayed the development of CTD. Specific cytokine analyses were performed. In **Chapter 9**, we showed that the gene expression of the cytokine EGF was significantly reduced and that the gene expression of TGF-β_1 was unmodified in the allografts with CTD. From day 50 and onwards gene and protein expression of the fibrogenic cytokine bFGF was increased in the CTD allografts. bFGF appeared to be produced by the infiltrated macrophages (**Chapter 10**).

Concluding Remarks and Perspectives

Studies in this thesis show that CTD develops in different organ transplants in a similar pattern. In both kidney and intestinal allografts with CTD, the predominant infiltrating cells are $CD4^+$ T cells and macrophages. In both organ transplants, the mild to moderate degree of intimal hyperplasia assumes that the concomitantly organ-specific lesions may not only be a consequence of ischemia due to vascular obliteration, but have an own entity in the process.

Despite the fact that CTD presents itself months to years after transplantation, the process is initiated at the moment of graft retrieval. Ischemia *per se* causes long-term functional and histological changes resembling those seen in allografts with CTD. In allografts, ischemia augments the alloimmune-mediated response. Since adhesion-molecule expression and cell infiltration is qualitatively identical during the first days in syngeneic and allogeneic grafts, which both develop CTD on the long-term, therapies to minimize this early ischemic injury are the first goal of treatment of CTD. At this moment, interference with transcriptional factors, such as NF-κB, that activate cytokine and adhesion molecule genes in response to ischemic injury, are a promising approach for the field of organ

transplantation. In an animal model of myocardial ischemic injury, the transfer of NF-κB oligodeoxynucleotides as a 'decoy' binds NF-κB and thus prevents activation of genes involved in ischemic injury. This led to reduction of myocardial infarction after reperfusion.[1] Interfering in the process at later stages is less effective because of the redundancy and Janus-faced character of several individual biologic factors, as clarified by knock-out studies.

Despite the role for ischemia in the process, alloimmunity is the predominant factor that induces and maintains the development of CTD. Allografts developed CTD in a much more rapid tempo than isografts.

Up to now, immunosuppressive therapies are basically related to T and B cell inactivation. In the chronic phase, however, macrophages appear to be the main producers of fibrogenic cytokines, such as bFGF and TGF-β. Strategies to eliminate or inactivate this cell population should be a point of interest. Also, a direct interference with the fibrogenic cytokines might be a future avenue. Antagonizing TGF-β or bFGF interrupts the fibrotic cascade.[2,3]

In order to identify patients at risk for CTD, the significance of gene polymorphisms of cytokines, growth factors and metabolic factors has to be further clarified. Recently, it has been found that a high-producer TGF-β_1 genotype in transplant recipients is associated with CTD.[4,5] Also, as shown in this thesis, donor organs may have their own genetic susceptibility to develop chronic lesions.

In addition to prevent CTD, to halt the process is another therapeutic strategy. Adding antihypertensive and antihyperlipedimic treatment to the immunosuppressive protocols appears meaningful to reduce the side effects of certain immunosuppressive drugs. In this view, stimulating the atrophied epithelium in small bowel and kidney transplants with CTD by mitogenic growth factors should be investigated.

To search for an optimal treatment, a further understanding of the pathophysiology is essential. Characterization of various cell populations, adhesion molecules, cytokines, and growth factors during the course of CTD, as performed in this thesis, should be followed by depletion or inactivation of the individual substances at different stages to understand their role in the process.

In conclusion, CTD has a multifactorial pathogenesis, in which both alloimmune specific and non-alloimmune specific factors are involved. A multiple treatment strategy at different stages in the process is likely the best design to overcome CTD.

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Samenvatting en Discussie

Ondanks de enorme verbetering van de 1-jaars overleving van orgaantransplantaten, met name door effectievere immunosuppressiva, is nà 1 jaar het transplantaatverlies gedurende de laatste 20 jaar vrijwel konstant gebleven; de gemiddelde overleving van een niertransplantaat bedraagt 7.5 à 9.5 jaar. Chronische transplantaat disfunctie (CTD) is de hoofdoorzaak van dit late transplantaat-falen. Klinisch uit CTD zich in een langzame achteruitgang van orgaanfunctie. Morfologisch is het pathogmonisch kenmerk van CTD circulaire arteriosclerose van intraparenchymale bloedvaten; daarnaast is er orgaanspecifieke pathologie, zoals bv. glomerulosclerose, tubulusatrofie en interstitiële fibrose in de nier.
De etiologie en de pathofysiologie van CTD zijn grotendeels onbekend. Er is geen therapie voorhanden om CTD te voorkomen. Retransplantatie is momenteel de enige effectieve behandeling.

De studies in dit proefschrift zijn opgezet om etiologische en pathofysiologische aspecten van CTD te onderzoeken, met als doel dat inzicht in het proces zal leiden tot nieuwe therapie strategiën.

Klinisch en dierexperimenteel onderzoek hebben aanwijzingen opgeleverd dat naast de immunologische respons van de ontvanger tegen het histo-incompatibele donor-orgaan ook niet-alloimmunologische factoren bijdragen aan CTD.
Hoofdstuk 2 geeft de resultaten weer van de eerste studie, waarbij het effect van langdurige koude ischemie op de ontwikkeling van het pathogmonisch kenmerk van CTD, intima hyperplasie, sec werd onderzocht. Om dit te realiseren werden aorta transplantaties uitgevoerd in een high responder (BN-WAG) combinatie in de rat. Zonder immunosuppressie ontstaat in dit model in 4 weken ernstige intima hyperplasie. Koude preservatie (4 °C) van de donor aorta's in UW-medium gedurende 24 uur veranderde de mate van intima hyperplasie niet. Ook in BN-BN syngene aorta transplantaten, die minimale intima hyperplasie hebben op 4 weken na transplantatie, had 24 uur preservatie geen invloed op de ontwikkeling.

De vraag bleef of koude ischemie invloed had op het parenchym van het transplantaat. Daarnaast zou de gevoeligheid van het endotheel voor ischemische schade afhankelijk kunnen zijn van locatie. Om de klinische orgaan

transplantatie na te bootsen, moest bovendien de invloed van immunosuppressie op ischemie worden onderzocht.
Vanuit een klinisch gezichtspunt, is onderzoek naar het effect van ischemie op CTD het meest relevant in een niertransplantatie model. Immers, niertransplantaten worden slechts 2 uur gepreserveerd in familie-nierdonatie programma's en tot meer dan 40 uur bij postmortale donaties.
Hoofdstuk 3 beschrijft de opzet van het niertransplantatiemodel en bespreekt de resultaten van het effect van de transplantatieprocedure op late schade in een bilateraal genefrectomeerde ontvanger. Dit werd onderzocht in een syngeen (BN-BN) model. De transplantatie procedure per se, waarbij inbegrepen een ischemietijd van 30 minuten, leidde niet tot verandering van nierfunktie en morfologie gedurende de studieperiode van 1 jaar.

Echter, syngene transplantaten die gedurende 24 uur werden gepreserveerd, ontwikkelden progressieve proteinurie vanaf week 18, en lieten op 1 jaar na transplantatie de histologische kenmerken van CTD zien: intima hyperplasie, glomerulopathie, en tubulaire atrofie. In de eerste dagen na transplantatie was er een toename van het aantal infiltrerende $CD4^+$ T-lymfocyten en macrofagen, wat samenging met een verhoogde expressie van ICAM-1 op het endotheel. Op 1 jaar na transplantatie was het aantal infiltrerende cellen nog steeds verhoogd vergeleken met de syngene transplantaten met minimale ischemietijd. In allogene transplantaten (BN-WAG) beinvloedde de 24 uurs preservatie van de donornier niet het ontstaan en beloop van CTD: Ischemische en niet-ischemische allotransplantaten die gedurende 3 weken behandeld werden met Cyclosporine om de acute afstoting te onderdrukken, ontwikkelden CTD in 26 weken op eenzelfde wijze. Net als in de ischemische syngene transplantaten bestond het type infiltrerende celllen voornamelijk uit $CD4^+$ T-lymfocyten en macrofagen, hoewel het aantal cellen hoger was, m.n. in de vroege fase. De discrepantie van het effect van langdurige ischemie tussen syngene- en allogene transplantaten lijkt gerelateerd aan de Cyclosporine therapie. Toediening van hetzelfde immunosuppressieve schema aan de ratten met syngene ischemische transplantaten leidde tot vermindering van koude ischemie geinduceerde CTD **(Hoofdstuk 4)**.

Een andere aanwijzing dat de behandeling met Cyclosporine de ischemische schade in allogene transplantaten remt, komt van bevindingen beschreven in **Hoofdstuk 5**. Zonder het gebruik van Cyclosporine versterkt ischemie de alloimmuun gemedieerde respons in de BN-WAG allotransplantaten.

Interstitiële infiltratie van voornamelijk $CD4^+$ T-lymfocyten, en tubulitis werden eerder waargenomen in de transplantaten die 24 uur gepreserveerd werden.

De experimenten die beschreven zijn in de **Hoofdstukken 2** en **6**, tonen aan dat chirurgie-gerelateerde factoren ook een invloed hebben op de ontwikkeling van CTD. In het syngene aortatransplantatie model had de manier van hechten van de anastomose invloed op de ontwikkeling van intima hyperplasie. In het middendeel van het 1 cm lange aortatransplantaat leidde doorlopend hechten tot meer intima hyperplasie dan onderbroken hechten. Rek van de vaatwand – uitgevoerd door een 8 mm lang aorta segment in een 12 mm gedissecteerde ontvanger te implanteren – had geen invloed op de mate van intima hyperplasie, maar verminderde wel de dikte van de media. In het syngene niertransplantatiemodel werd aangetoond dat de uretero-neocystostomie als reconstructie-techniek van de tractus urinalis, bijdraagt aan lange-termijns funktionele en histologische nierafwijkingen. Daar de intrapelvicale druk verhoogd was, lijkt een partitiële obstructie de oorzaak van nierschade.

Hoofdstuk 7 beschrijft de effecten van het transplanteren van donor nieren van verschillende rattenstammen in één histocompatibele F_1-ontvanger. Op deze wijze werden de transplantaten blootgesteld aan gelijke bloeddruk, metabole en hormonale omgeving. De ACI rattenstam, resistent tegen hypertensie-geïnduceerde nierschade, de hypertensieve FHH rat, gevoelig voor hypertensie-geïnduceerde nierschade, en de (ACI x FHH) F_1. De funktie en morfologie van een F_1 of een ACI niertransplantaat in de normotensieve F_1 ontvanger was gelijk; Bovendien verschilde deze niet van hun unilateraal genefrectomeerde ACI en F_1 controles op 16 weken na transplantatie. Daarentegen ontwikkelde het FHH niertransplantaat een progressief verlies van funktie en toonde histologische kenmerken van CTD. Uit deze studie kunnen we concluderen dat de genetische gevoeligheid van de donornier voor schade een factor is in de etiologie van CTD.

Hoofdstuk 8 beschrijft de ontwikkeling van een reproduceerbaar CTD-model na dunne darm-transplantatie en de daarmee gepaard gaande funktionele, histologische en inflammatoire veranderingen. Funktionele afwijkingen en histologische kenmerken ontwikkelden op dag 50 na transplantatie. Het type infiltrerende cellen in het transplantaat bestond voornamelijk uit $CD4^+$ en $CD8^+$ T-lymfocyten en macrofagen. B-lymfocyten en NK-cellen lijken niet betrokken in het proces. Cyclosporine therapie vertraagde de ontwikkeling van CTD. In dit model werden de expressie van cytokinen onderzocht:

Hoofdstuk 9 toont aan dat de genexpressie van het cytokine EGF significant

verminderd was in allotransplantaten met CTD. De expressie van TGF-β_1 was ongewijzigd.
Vanaf dag 50 na transplantatie was de gen- en eiwitexpressie van het fibrogene cytokine bFGF verhoogd in de dunne darmtransplantaten met CTD. Uit deze studie lijkt de macrofaag de voornaamste bron van bFGF te zijn (**Hoofdstuk 10**).

Conclusies en Vooruitzichten

De studies beschreven in dit proefschrift tonen aan dat de ontwikkeling van CTD in verschillende orgaan transplantaten op analoge wijze verloopt. Zowel in nier als dunne darm allotransplantaten met CTD zijn $CD4^+$ T-lymfocyten en macrofagen de belangrijkste typen infiltrerende cellen. De milde tot matige intima hyperplasie in beide orgaantransplantaten veronderstelt dat de gelijktijdig optredende orgaan-specifieke lesies niet alleen het gevolg zijn van ischemie door vasculaire obliteratie, maar ook een eigen entiteit in het proces hebben.

Ondanks het feit dat CTD pas maanden tot jaren na transplantatie manifest wordt, begint het proces op het moment van orgaanuitname. Ischemie sec veroorzaakt funktionele en histologische afwijkingen op de lange-termijn, die overeenkomen met de afwijkingen in allo-transplantaten met CTD. In allo-transplantaten versterkt ischemie de alloimmuun-gemedieerde respons.

Daar de expressie van adhesie-molekulen en celinfiltratie kwalitatief gelijk is in syngene en allogene transplantaten gedurende de eerste dagen na transplantatie, zijn therapieën gericht op het minimaliseren van deze ischemische schade het eerste doel van behandeling van CTD. Op dit moment lijkt interferentie met transcriptie-factoren, zoals NF-κB, die cytokine en adhesiemolecuul genen activeren in respons op ischemische schade, een veelbelovende strategie. In een diermodel van ischemische hartschade voorkomt het overbrengen van NF-κB oligodeoxynucleotiden als 'lokeend' voor NF-κB de activatie van genen die betrokken zijn bij ischemie. Dit leidde tot een vermindering van het myocardinfarct na reperfusie. Latere interferentie in het CTD-proces is minder effectief vanwege het pleiotrope karakter van veel biologische factoren, zoals aangetoond met knockout-studies.

Ondanks de rol voor ischemie in het proces is alloimmuniteit de belangrijkste factor die de ontwikkeling van CTD in gang zet en onderhoudt. Allogene transplantaten ontwikkelden sneller CTD dan syngene transplantaten.

Tot op heden zijn de immunosuppressieve therapien gericht op T- en B cel inaktivatie. In de chronische fase, echter, zijn de macrofagen de producenten van fibrogene cytokinen zoals bFGF en TGF-β. Eliminatie of inaktivatie van deze celpopulatie behoort een serieus doel te zijn. Ook direkte interferentie met de fibrogene cytokinen is een potentiële invalshoek. Het antagoneren van TGF-β of bFGF onderbreekt de fibrotische cascade.

De betekenis van genpolymorfisme van cytokinen, groeifactoren en metabole factoren dient verder opgehelderd worden teneinde patienten te identificeren die een verhoogd risico hebben op het ontwikkelen van CTD. Recent is aangetoond dat een high-producer TGF-β_1 genotype in transplantatie-patienten geassocieerd is met CTD. Daarnaast kunnen ook de donor organen hun eigen genetische gevoeligheid hebben om schade te ontwikkelen, zoals is aangetoond in deze dissertatie.

Naast het voorkomen van CTD is het remmen ervan een optie. Het toevoegen van antihypertensieve en antihyperlipidemische medicatie aan de immunosuppressive protocollen lijkt zinvol om de nevenwerkingen van bepaalde immunosuppressiva te reduceren. Vanuit dit gezichtpunt is het stimuleren van geatrofieerd epitheel in dunne darm- en niertransplantaten met CTD met mitogene groeifactoren een mogelijkheid.

Om de optimale therapie te vinden, is verdere studie van de pathofysiologie essentieel. Het karakteriseren van verscheidene cel populaties, adhesiemolekulen, cytokinen en groeifactoren tijdens de ontwikkeling van CTD, zoals uitgevoerd in dit promotieonderzoeks, behoort gevolgd te worden door depletie en inactivatie van de individuele celtypen en factoren tijdens verschillende stadia om zo hun preciese rol te begrijpen.

In conclusie: CTD heeft een multifactoriële pathogenese, waarbij zowel alloimmuun specifieke als niet-alloimmuun specifieke factoren betrokken zijn. Een multipele behandelingsstrategie tijdens diverse stadia in het proces is waarschijnlijk de beste opzet om CTD te overwinnen.

Appendices

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Nawoord

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Curriculum Vitae

De auteur van dit proefschrift werd op 10 februari 1969 geboren te Delft. Voortgezet wetenschappelijk onderwijs werd genoten op het Van Lodenstein College te Amersfoort van 1981 tot 1987. Na een jaar op de Hogeschool voor Journalistiek in Amersfoort begon hij in 1988 de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam. Gedurende de doctoraalfase verrichtte hij onderzoek op het Laboratorium voor Experimentele Chirurgie naar xenogene transplantatie van eilandjes van Langerhans (dr. R.L. Marquet). De coschappenperiode werd afgesloten met een stage van vijf maanden in het Deborah Retief Memorial Hospital in Mochudi, Botswana (drs. A.A. Hogewoning). In december 1994 behaalde hij zijn artsexamen cum laude. Vervolgens werkte hij als officier-arts bij de Koninklijke Landmacht ter vervulling van zijn dienstplicht. Sedert september 1995 werkt hij als assistent in opleiding (AIO) op het Laboratorium voor Experimentele Chirurgie aan de Erasmus Universiteit (prof.dr. J.Jeekel). De begeleiding van het onderzoek naar de etiologie en pathofysiologie van chronische transplantaat disfunctie was in handen van dr. J.N.M. IJzermans en dr. R.W.F de Bruin. Met het verkrijgen van een stipendium van het Collegium Chirurgicum Neerlandicum werkte hij tussentijds (september 1996 tot april 1997) op het Department of Medicine aan de Monash University in Melbourne, Australia (prof.dr. N.M. Thomson). In dit reeds opgezette samenwerkingsverband nam hij deel aan de studie naar de pathofysiologie van chronische transplantaat disfunctie in dunne darmtransplantaten, onder de dagelijkse begeleiding van dr. A.N. Stein-Oakley. Hij begint 1 juli aanstaande aan de opleiding tot chirurg in het Diaconessenhuis te Eindhoven (dr. W.J. Prakken) en zet deze voort in het Academisch Ziekenhuis Maastricht (prof.dr. G. Kootstra).

List of Abbreviations

ACI	August x Copenhagen
bFGF	basic fibroblast growth factor
BN	Brown Norway
CsA	Cyclosporine A
CMV	cytomegalovirus
CR	chronic rejection
CTD	chronic transplant dysfunction
EGF	epidermal growth factor
FHH	Fawn Hooded
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HLA	human leucocyte antigen
ICAM	intercellular adhesion molecule
IFN	interferon
IGF	insulin growth factor
IL	interleukin
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
MΦ	macrophages
PDGF	platelet derived growth factor
RT-PCR	reverse transcriptase-polymerase chain reaction
sd	standard deviation
SEM	standard error of the mean
TGF	transforming growth factor
TNF	tumor necrosis factor
UW	University of Wisconsin
WAG	Wistar Agouti