Endothelial Dysfunction and Denudation in Rat Aortic Allografts

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Abstract—Clinical evidence suggests that early endothelial cell (EC) dysfunction may predict the development of graft vascular disease. We wished to assess the early functional and morphological changes in the graft endothelium in a commonly used animal model of graft vascular disease, the rat aortic interposition allograft model. To assess graft EC function, regulation of vascular tone by ECs was monitored in aortic rings from grafts harvested at various times after transplantation (Tx). EC morphology was assessed by silver staining, which was followed by en face inspection of the luminal side of the grafts. Acetylcholine-induced EC-dependent vasorelaxation was reduced in allografts at post-Tx days 7 and 14, whereas in syngeneic grafts EC-dependent relaxation was unaffected at any time after Tx. In separate grafts collected at the same time points, massive leukocyte adhesion at post-Tx day 7 and EC denudation at days 14 and 28 were evident in allografts but not in syngeneic grafts. At post-Tx day 56 (a time at which vessel wall remodeling is pronounced in this model), an intact EC layer covered the grafts. EC dysfunction and morphological changes were prevented by immunosuppression of recipient rats with cyclosporine. Our study shows that Tx-induced EC dysfunction in rat aortic allografts can be observed within 1 week of Tx in rat aortic allografts and that this is occurring concomitantly with enhanced leukocyte adhesion to the graft ECs. These changes occur before any other morphological or functional changes described thus far in this model and appear to be immune-driven. Taken together, these results show that Tx-induced early EC dysfunction, as described in patients, may be studied in the model of rat aortic Tx. (Arterioscler Thromb Vasc Biol. 2001;21:67-73.)

Key Words: endothelium ■ vasorelaxation ■ graft ■ rejection ■ aorta

The development of graft vascular disease (GVD) is a leading cause of long-term graft failure. GVD is mainly characterized by the accelerated development of concentric atherosclerotic lesions in arteries of solid organ grafts, leading to impaired blood perfusion and functional deterioration (see reviews1,2). Although the exact pathogenesis of GVD remains to be established, several lines of evidence suggest that it may be initiated by an early injury at the level of the graft endothelial cells (ECs),3,4 inasmuch as these cells are the first donor cells to be encountered by recipient leukocytes. Supportive evidence is provided by a clinical study demonstrating that early endothelial dysfunction, seen within 15 days after transplantation (Tx), predicts the development of intravascular ultrasound–detectable Tx coronary artery disease at 1 year after heart Tx.5 In addition, the degree of early activation of arterial/arteriolar endothelium, as defined by intercellular adhesion molecule-1 or human leukocyte antigen-DR expression, predicts the rate of development of coronary artery disease and the risks of graft failure.6

In the present study, we wished to assess the function of graft endothelium at early post-Tx time points and to monitor morphological changes in graft ECs. We used a model of rat aortic Tx with the stringent (full major histocompatibility complex mismatch) strain combination dark agouti (DA, RT1a) and Lewis (RT1b). Vessel Tx models have been described as tools to study the development of vascular lesions mimicking GVD.7–10 However, they have been mainly characterized in terms of Tx-induced histopathological changes, such as neointimal formation and cellular infiltration, and very little is known about the consequences of Tx on the function of the vascular wall. In a recent study, we showed that although rat aortic allografts maintain their function of blood conductance for weeks after Tx, they lose their functional smooth muscle cells (SMCs) within 14 days after Tx, suggesting an acute rejection of SMCs occurring before any visible signs of vascular remodeling (not seen before 4 weeks after Tx).11 To our knowledge, graft endothelial dysfunction has not been assessed in rodent models of Tx as yet. Therefore, in the present study, we focused our attention on the functional and morphological consequences of Tx on the graft endothelium.

To this end, endothelium-dependent relaxation to acetylcholine (ACh) was assessed in allografts as well as in syngeneic grafts (DA to DA) collected at different time points.
after Tx. Also, en face silver staining was performed to better characterize the morphological changes taking place at the level of the endothelium. Results show that in aortic allografts, endothelial dysfunction, leukocyte adhesion, and morphological changes in the ECs occur early after Tx and before the loss of functional SMCs and weeks before manifest vascular remodeling. We conclude that it is possible to assess Tx-induced early EC dysfunction in rat aortic allografts and that such a model could be used to assess the protective effects of pharmacological agents.

Methods

Reagents

AgNO₃, CoBr₂, and NH₄Br were obtained from Fluka. Solutions used during the experiments were prepared freshly every day. Glutaraldehyde (25% stock solution, Merck) was diluted to a final concentration of 2.5% in PBS (Dulbecco’s PBS) on the day of the experiment.

Animals

Rats (inbred strains RT1⁺ and RT1⁻) were obtained from Harlan (Zeist, The Netherlands). They were allowed unrestricted access to food and water before and after the operation. Handling, care, and experimentation were performed in compliance with the Swiss federal law for animal protection.

Tx Experiments

Recipient rats (250 to 300 g) were anesthetized by inhalation with a mixture of N₂O/O₂ (70%/30%) combined with isoflurane (3.5% to 3% for maintenance), placed on a thermoregulated blanket to keep rectal temperature at 37°C, and orthotopically transplanted with a 1-cm-long segment of syngeneic abdominal aorta with end-to-end sutures. After retrieval, each donor aorta was kept for a 10-minute maximum in cold saline (4°C) before Tx. For each graft, the surgery time, during which the graft is exposed to body temperature, did not exceed 30 minutes.

For the animals treated with cyclosporine (7.5 mg/kg Neoral, Novartis Pharma AG) or placebo, treatment started a few hours after Tx Experiments (after the animals had recovered from anesthesia) and was performed by daily gavage (200 L/100 g body wt) for 28 days. With this dosing regimen, cyclosporine plasma values were 35 ng/mL at 8 hours. Trough levels at 24 hours (before administration of the next dose) were 6 ng/mL. Similar results were previously reported in the rat. With this dosing regimen, cyclosporine plasma values were 35 ng/mL at 8 hours. Trough levels at 24 hours (before administration of the next dose) were 6 ng/mL. Similar results were previously reported in the rat.

Assessment of Endothelium-Dependent Vasorelaxation

At 3, 7, and 14 days after Tx, rats were euthanized by cervical dislocation and then exsanguinated by carotid artery transection. Abdominal aortas were rapidly collected, put in ice-cold Krebs’ buffer, and cleaned of connective tissue. From each recipient, nontransplanted (native) and transplanted (graft) tissues were harvested. Rings of 2 to 3 mm were cut in the middle of the grafts (=2 mm away from both ends used for the anastomoses). The rings were then mounted in standard organ baths (Schuler, Hugo Sachs Elektronik) filled with Krebs’ buffer (composed of [mmol/L] NaCl 119, KCl 4.7, CaCl₂ 1.25, MgSO₄ 1.17, KH₂PO₄ 1.18, NaHCO₃ 25, and glucose 11) maintained at 37°C, and continuously bubbled with a 95% O₂/5% CO₂ mixture. Resting tension was adjusted to 500 mg. Tension was measured with an isometric force transducer (Statham) connected to amplifiers and a polygraph recorder (Rikadenki).

After being washed 3 times for 60 minutes, the vessels were sensitized with K⁺ (40 mmol/L), and once the constrictor response had stabilized, ACh (1 µmol/L) was added to the bath to assess the level of endothelium-dependent vasorelaxation. Preparations were washed again 3 times before being preconstricted with phenylephrine (Phe, 10 µmol/L). When the contraction reached a steady state, ACh (10 µmol/L) was added to the bath to assess the endothelium-dependent vasorelaxation. In some experiments, the relaxing effects of 8-bromo-cGMP (30 µmol/L) and forskolin (10 µmol/L) were assessed in Phe-preconstricted vessels to determine whether the SMC relaxation transduction pathways, ie, cGMP- and cAMP-dependent signaling, were functional after Tx.

Perfusion Fixation and Silver Staining

The method of in situ silver staining was performed as previously described with minor modifications. Briefly, rats were euthanized at post-Tx days 1, 3, 7, 14, 28, and 56 with an overdose of anesthetic (pentobarbital sodium, 60 mg/kg IP). After thoracotomy, a cannula was inserted through the left ventricle into the aortic arch and secured with a ligature. An orifice was cut into the wall of the left ventricle, and the animal was perfused with PBS at a pressure of 150 mm Hg for 6 minutes, followed by glutaraldehyde fixative (2.5% glutaraldehyde in PBS) for 5 minutes. After another 3-minute perfusion with PBS, the staining solutions (sequentially, 0.1% AgNO₃, and then 1% HN₄Br+3% CoBr₂, with the latter 2 as a mixture) were perfused for 1 minute each with 1-minute washes with PBS between the staining solutions. The graft and adjacent host aortas were excised carefully, cut open longitudinally under a dissecting microscope, and pinned down on a plastic mold, with the endothelium facing up. Fixation was continued for ~2 hours under a lamp to intensify the staining. The fixed and stained tissue was dehydrated in graded ethanol and mounted between glass slide frames in Epon (Fluka) and cured overnight at 60°C. This procedure yields permanent tissue preparations.

Data Analysis

Vasorelaxation is expressed as percent decrease from the preconstriction level of each preparation. Results are expressed as mean±SEM of n experiments. ANOVA was performed for statistical analysis with the use of SigmaStat software (SPSS Inc). A level of P<0.05 was considered significant.

The staining quality was assessed on the host aorta excised together with each transplant. Vessels in which the staining quality was not adequate because of inappropriate perfusion were discarded and not included in the analysis.

Scoring systems were used to assess the severity of endothelial denudation (0, no denudation; 1, spots of denudation about the size of a few ECs; 2, larger spots of denudation of several cell diameters; 3, large areas of denudation; and 4, most of the graft denuded) as well as the degree of leukocyte adhesion (0, no adhesion; 1, small spots of adhesion scattered in parts of the graft; 2, adhesion scattered all before and after the graft; and 3, dense adhesion covering most of the graft). For analysis, each sample was coded and scored by an independent observer blinded to the experimental code.

Results

Endothelium-Dependent Relaxation

As shown in Figure 1, stimulation of vessel rings with 10 µmol/L ACh produced 30% relaxation in Phe-preconstricted native aorta (Figure 1A). A similar ACh-mediated relaxation was also seen in rings of syngeneic aortic grafts collected at days 3, 7, 14, and 28 after Tx.

In allografts collected at day 3 after Tx, ACh-mediated relaxation was similar to that in native aorta (Figure 1B). In contrast, in aortic allografts collected at days 7 and 14 after Tx, ACh-mediated relaxation was severely reduced (by 80% to 90%) compared with relaxation in native aorta (Figure 1B), although the relaxation responses to 8-bromo-cGMP and forskolin were maintained (Figure 2). This indicates that the SMC-dependent pathways leading to vasorelaxation are functional, whereas the endothelium-dependent relaxation is severely impaired. We have previously shown that at day 28 after Tx, aortic allografts from nonimmunosuppressed rats are...
unable to constrict in response to Phe. Therefore, the assessment of endothelium-dependent relaxation was impossible in these animals. Nevertheless, in allografts collected at day 28 after Tx from animals treated daily with cyclosporine (7.5 mg·kg⁻¹·d⁻¹ Neoral), the ability to constrict in response to Phe and to relax in response to ACh was maintained (Figure 1B). Similar results were obtained in cyclosporine-treated allografts collected at day 14 after Tx (data not shown).

Assessment of EC Morphology by Silver Staining

To assess the morphology of graft endothelium over the entire length of the graft, we used the technique of silver staining followed by en face inspection of each graft with adjacent host aorta. This staining outlines the EC borders in situ perfusion-fixed and stained rat aortic grafts. Representative examples of the staining patterns obtained with allografts are illustrated in Figure 3 (for the examples with syngeneic grafts, please refer to Figure I, which can be accessed online at http://atvb.ahajournals.org). The morphological appearance of the endothelium in allografts as well as syngeneic grafts was quantified by means of a scoring system, and the results obtained after blinded analysis are summarized in Figure 4.

At post-Tx day 1, allografts and syngeneic grafts showed a similar extent of endothelial injury (most likely due to the trauma associated with surgery and/or ischemia/reperfusion injury). Severe endothelial denudation was seen at the anastomoses, whereas small spots of denudation were disseminated over the entire length of the grafts. At post-Tx day 3, endothelial injury was still seen at the level of anastomoses, but allografts and syngeneic grafts showed intact EC layers, but with altered orientation of ECs compared with the host aorta. Similar to the findings on day 1, spots of leukocyte adhesion were rarely (in 1 of 6 samples) observed in these tissues.

At day 7 after Tx, allografts and syngeneic grafts had almost intact endothelial borders, even at the level of anastomoses. In contrast to syngeneic grafts, scattered leukocyte adhesion covering >50% of the graft surface was observed in allografts. Figure 5 illustrates the very clear demarcation between the allograft and the host endothelium at this time point after Tx. At days 14 and 28 after Tx, syngeneic grafts showed normal EC outlines. In contrast, endothelial denudation was substantial in allografts, with large spots of dark silver deposits devoid of ECs. Also, leukocyte adhesion in the non-denuded areas appeared more severe than at day 7 and was scattered over the entire length of the grafts.

It was noted that at day 28 after Tx in allografts from nonimmunosuppressed animals, reendothelialization occurred at the level of the anastomoses with intact ECs devoid of adhering leukocytes (except at the leading edge; see Figure 3E) progressing toward the center of the graft. On the basis of this morphological pattern, it can be assumed that these cells may be of host origin.

At day 56 after Tx, allografts presented a morphologically intact endothelium on their entire surface with only rare adherent leukocytes (Figure 3F). The effect of immunosuppression on the endothelial integrity was illustrated in allografts treated daily with 7.5 mg·kg⁻¹·d⁻¹ cyclosporine and collected at post-Tx day 28. In these animals, the endothelial layer was intact without any signs of leukocyte adherence (please refer to Figure II, which can be accessed online at http://atvb.ahajournals.org). Similar
results were observed in cyclosporine-treated allografts collected at day 14 after Tx (data not shown).

**Discussion**

In the present study, we wished to develop a model that would allow the functional and morphological assessment of graft ECs during the early phase of graft rejection. We used a rat aorta interposition graft model, a model widely used in experimental Tx often in the context of chronic rejection. We have previously shown that in this model, the stringent strain combination (DA to Lewis), functional SMCs are lost within 14 days after Tx in the absence of immunosuppression. The results of the present study show that as early as 7 days after Tx in the absence of immunosuppression, allografts have completely lost the endothelium-dependent regulation of vascular tone, as demonstrated by the absence of vasorelaxant response to ACh. At the same time after Tx, although the endothelium appeared morphologically intact, high numbers of leukocytes adhering to the luminal side of the ECs were observed. This suggests that the Tx-induced endothelial dysfunction may, in the present model, be driven primarily by the EC-leukocyte interactions. Leukocyte adhesion and the loss of EC-dependent relaxation are most likely the consequences of processes involved in the allogeneic response because syngeneic grafts, as well as allografts from cyclosporine-treated rats, maintained their ability to respond to ACh and were devoid of adhering leukocytes up to 28 days after Tx. Taken together, these results suggest that the present model of rat aortic Tx can be used to monitor Tx-induced endothelial dysfunction. This occurs early after Tx (at approximately post-Tx day 7 in this strain combination) and precedes the loss of SMC function.

To our knowledge, this is the first report demonstrating that in the chronology of events leading to rejection of rat aorta allografts, EC dysfunction occurs early after Tx and, together with EC-leukocyte interaction, is the first parameter to indicate rejection. EC destruction (the present study and others) and SMC loss follow. The development of a neointima analogous to that in clinical GVD is a late event in this model.

Previous experiments addressing the fate of ECs in vessel transplant models were restricted to the careful histological analysis of allografts and did not take into account the graft EC functionality. Plissonnier et al and Gohra et al have documented that the endothelium of vessel allografts after Tx is subjected to a first injury, which is most likely due to mechanical trauma and reperfusion injury, followed by a healing process and by a secondary injury, most likely depending on the alloimmune response. These events were described as taking place within 2 to 3 weeks after Tx and being followed by the regeneration of an intact endothelium, most likely of recipient origin. Our observations are in agreement with those 2 studies, inasmuch as we also observed EC damage at post-Tx day 1, which was most likely of traumatic origin because it was observed in allografts and syngeneic grafts. A regeneration process between days 3 and 7 restored the endothelial lining in allografts and syngeneic grafts. A secondary EC injury was observed at day 7, beyond which it was most likely immune-driven because it did not occur in syngeneic grafts and because it was completely prevented in allografts by cyclosporine treatment. We also observed a complete regeneration of the graft endothelial layer at 8 weeks after Tx. It is interesting to note that the primary trauma provoked by the surgical procedure (mainly at the level of anastomoses but also focally throughout the
graft) was not associated with any detectable EC dysfunction, inasmuch as compared with native aortas, allografts and syngeneic grafts collected on day 3 after Tx showed similar responses to ACh. Once this primary insult has healed (at 7 days after Tx), ECs in allografts are able to interact with leukocytes but are no longer able to elicit relaxation of SMCs in response to ACh. This confirms that the loss of the EC-mediated relaxing effect of ACh in these allografts is not of traumatic origin but is most likely due to immune-driven impairment of the EC-dependent mechanisms involved in the control of SMC tone.

The role of ECs in the control of SMC tone is well established and is mediated by the so-called endothelium-derived relaxing factors (EDRFs), including NO, prostaglandin I2, and a still unidentified hyperpolarizing factor (see review20). Hence, an impaired relaxation to ACh, as seen in the present study, may reflect a reduced availability of EDRF and/or alterations in the relaxation-transduction pathways within the SMCs. Our results demonstrate that the 2 major transduction pathways controlling the SMC relaxation, ie, cGMP- and cAMP-dependent pathways,21 are fully functional, inasmuch as they both respond to direct stimuli such as 8-bromo-cGMP and forskolin, respectively (see Figure 2). Therefore, it appears that in rat aorta allografts, a decrease in the availability of EDRF is responsible for Tx-induced loss of ACh-mediated relaxation. Similar observations were reported by clinical studies showing that although coronary vessels of cardiac transplant patients with Tx coronary artery disease were not responding to EC-dependent vasodilators, they could still dilate in response to EC-independent dilators, such as nitroglycerin or adenosine.5,22,23 The molecular pathways leading to a reduction in EDRF availability in allografts are not fully understood at present. Additional experiments are necessary to determine whether this is due to a reduced production or an accelerated degradation of the mediators released by ECs. A possible mechanism could be a dysfunction at the level of endothelial G proteins, as recently observed in the coronary bed of pig heart allografts.24 Whatever the case, it is most likely an early consequence of the EC-leukocyte interaction, which (as demonstrated by the present study) occurs just before EC denudation.

From the present study, the leukocytes responsible for the activation and subsequent destruction of the graft ECs cannot be identified, but the mechanisms involved in such a secondary Tx-induced EC denudation in vessel allografts are under
investigation. EC-specific allorestRICTED cytotoxic T lymphocytes have been described in several experimental systems and have been demonstrated by using T-cell lines derived from human allotransplant recipients. Very recently, they have been generated in vitro. In addition, microvascular EC sloughing has been observed in models of Tx. Therefore, it is tempting to speculate that EC-specific cytotoxic T lymphocytes may be involved in the processes described above.

Interestingly, after denudation, aorta allografts observed 8 weeks after Tx have regained an intact EC layer. These cells most likely are from the recipient origin, because morphological signs of EC regrowth were observed on the edges of allografts at post-Tx day 28. Similar observations were also reported by other investigators. It remains to be established whether this reendothelialization process is generating a functional endothelium, ie, is able to respond to ACh. We have been unable to clarify this point because grafts collected at days 28 and 56 after Tx could no longer be preconstricted with vasoconstricting agents, as a consequence of the loss of functional SMCs. Therefore, at these late post-Tx times, rat aortic allografts appear as nonfunctional conduits, which are not able to respond to vasodilating or vasoconstricting agents but are still capable of maintaining systemic blood circulation in vivo. In these conditions, the loss of endothelium-dependent control of the vascular tone is expected to have no impact on the lumen size of the vessel grafts. It has indeed been reported that vessel allografts, such as rat carotid arteries and aortas, remain fully patent up to 8 weeks after Tx, with a bulging mainly at their center observed in vivo by MRI or by histology/morphometry, suggesting arterial wall shrinkage or aneurysm formation.

As demonstrated in our previous study, neointimal formation starts at the edges of DA-to-Lewis rat aortic allografts only after post-Tx day 28, and full coverage of grafts by a thick neointima is usually observed at post-Tx day 56. It was proposed that neointimal formation could progress from both edges to the center of the grafts and was most likely due to the proliferation of SMCs of donor origin. It is tempting to think about a possible relationship in the present model between the degree of neointimal formation observed at 56 days after Tx and the loss of EC function observed at day 7 after Tx. Such a correlation has been demonstrated by longitudinal studies in heart-Tx patients developing GVD. Because such studies are difficult to reproduce in experimental models, proof of correlation may be provided by pharmacological agents that can specifically prevent Tx-induced EC functional loss. It can be assumed that therapeutically preventing or minimizing graft EC dysfunction would result in improved long-term graft outcomes. Despite the obvious differences between the clinical reality of GVD and the animal model used in the present study, at least some of the mechanisms instrumental in the pathology may be the same, and gaining insight into the pathophysiology in the animal model may help understand processes in clinical Tx rejection.

References


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