Transplant arteriosclerosis
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Chapter 5

The Rate of Development of Transplant Arteriosclerosis is determined by Recipient Non-MHC encoded Genes

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Chapter 5

Abstract

Background. Chronic transplant dysfunction (CTD) is the major complication in long-term survival of solid organ transplants. Histologically, CTD is characterized by transplant arteriosclerosis (TA), consisting of concentric myointimal proliferation (vascular smooth muscle cells) resulting in the development of an occlusive neointima in the arterial structures of the graft. Development of TA apparently is multifactorial and alloantigen-dependent as well as -independent factors have been identified. However, also genetic variation between transplant recipients appears to be involved in the process of TA development.

Methods. In this study we analyzed the contribution of host MHC and non-MHC encoded genes on the development of TA in aortic allografts using different rat strains. Aortic allografts were transplanted in Lew, BN and F344 rats as well as in the MHC-congenic Lew.1N and BN.1L rat strains. Moreover, allografts were transplanted in (Lew
\[\text{BM} \rightarrow \text{BN}\) and (BN
\[\text{BM} \rightarrow \text{Lew}\) bone marrow chimeric rats. Grafts were explanted at different time points after transplantation and the severity of TA was determined by morphometry.

Results. Rats bearing the BN non-MHC haplotype (BN and BN.1L) appeared to be high TA responders compared to rats bearing the Lew non-MHC haplotype (Lew and Lew.1N). Differences were primarily observed in the kinetics of TA development rather than in the degree of TA developing eventually. Allografts transplanted in F344 and bone marrow chimeric rats suggested that the high TA responsiveness observed in BN and BN.1L rats is most likely determined by factors other than the immunologic responder-status.

Conclusion. The kinetics of TA development is predominantly determined by host non-MHC encoded determinants, indicating that the rate of development of TA is genetically controlled. Which factors are responsible for this effect remains unclear. Since genetic differences between organ transplant recipients appear to determine the rate of TA development, identification of the gene products responsible for this phenomenon will be essential both in tracing patients who are at risk to develop TA fast, as well as in developing new strategies to prevent or treat TA.
Introduction

To date, the development of chronic transplant dysfunction (CTD) is the primary cause of loss of solid organ transplants after the first postoperative year. Unfortunately, an adequate strategy to prevent or treat CTD is still lacking\(^1\,^2\). The most characteristic histologic feature of CTD is transplant arteriosclerosis (TA) which is characterized by a generalized, concentric intimal thickening predominantly consisting of vascular smooth muscle cells intermingled with some inflammatory cells (T cells and macrophages)\(^3\,^4\). TA has generally been accepted as the main cause of progressive deterioration in graft function leading to death or the need for retransplantation eventually. The pathogenesis of TA appears to be multifactorial, though alloreactivity of the host against the graft seems to be the most important factor contributing to the development of TA, and several alloantigen associated risk factors (e.g., histoincompatibility and acute rejection) have been identified. However, also alloantigen independent factors like ischemia/reperfusion injury, donor brain death and viral infections seem to contribute to the development of TA\(^1\,^5\).

Among transplant recipients, transplanted with similar HLA-incompatible grafts and receiving similar immunosuppression, variation exists in both the rejection rate and long-term outcome. This variation has not been fully explained, but data indicate that different individuals might display different immune responses against an allograft (different immunologic responder status)\(^6\). One possible candidate responsible for such individual variation is genetic variance in the regulation of cytokine gene expression. Thus accelerated onset of TA in human cardiac allografts as well as CTD in lung allografts has been found to correlate with genetically predisposed high TGF-\(\beta\) production\(^7\,^9\). In addition, individual variation in other, non-immune related, yet unidentified factors, might also contribute in determining the rate and severity of TA after solid organ transplantation.

To gain further insight in the contribution of recipient MHC and non-MHC (among others the immunologic responder status) encoded determinants, we here analyzed the development of TA in aortic allografts using different rat strains as donors and recipients. Using morphometry we showed that Lew and BN rats used as hosts displayed significant differences in primarily the kinetics of TA development (low vs. high TA responders, respectively). Aortic transplantation performed in congenic BN and Lew rat strains revealed that these differences were independent of the MHC-haplotype of the host. Since Lew and BN rats represent strains with a different immunologic responder status (Th1-prone vs. Th2-prone)\(^10\), we wondered whether the Th2-skewed responder status of BN rats contributed to the high TA responsiveness. However, F344 hosts (Th2-prone responder) developed only mild TA, suggesting that factors other than recipient’s immunologic responder status are involved in determining the onset and severity of TA after allogeneic aorta transplantation in rats. This was further strengthened by results obtained from allografts transplanted in bone marrow chimeric rats.
Materials and Methods

Animals
Specified pathogen free male Lew (RT-1\(^a\)), BN (RT-1\(^n\)), F344 (RT-1\(^{11}\)) and DA (RT-1\(^i\)) rats were obtained from Harlan (Zeil, The Netherlands). MHC congenic Lew.1N (RT-1\(^n\)) and BN.1L (RT-1\(^l\)) rats were obtained from the central animal facilities of Maastricht University. Rats, 8-10 weeks of age, were kept under clean conventional conditions and were fed standard rat chow and acidified water ad libitum. All animals received humane care in compliance with the Principles of Laboratory Animal Care (NIH Publication No.86-23, revised 1985) and the Dutch Law on Experimental Animal Care.

Aorta transplantation
Aortic allografts (10-12 mm) were transplanted as described previously\(^{11}\). Briefly, the abdominal aorta between the left renal artery and the bifurcation was removed from the donor rat and perfused with saline to remove blood cells. Subsequently, the aortic graft was orthotopically transplanted into the recipient rat via end-to-end anastomosis using 9-0 nylon suture. Total ischemic time was consistently less than 30 minutes during which the grafts were kept in ice-cold saline.

Experimental groups
To study whether the kinetics and the severity of TA differ among different donor/recipient rat strain combinations, aortic transplantations were performed in the combinations as listed in Table 1. First we analyzed the development of TA in the fully MHC incompatible BN to Lew (groups 1-4) and the reciprocal Lew to BN (groups 5-8) strain combinations (Table 1A). Aortic allografts were explanted 4, 8, 12 and 24 weeks after transplantation and subsequently processed for histologic and morphometric analysis. BN isografts (group 9) served as controls and were explanted 3 months after transplantation.

To study whether the differences observed in the above mentioned strain combinations were determined by recipient’s MHC or non-MHC encoded determinants, additional aortic allografting was performed using MHC-congenic Lew.1N and BN.1L rats (Table 1B). Lew.1N congenic rats bear the BN-derived MHC haplotype on the Lew (non-MHC) genetic background, whereas BN.1L congenic rats bear the Lew derived MHC haplotype on the BN (non-MHC) genetic background. Transplantations were performed in the MHC-incompatible, but non-MHC identical, BN to BN.1L (groups 10 & 11) and Lew to Lew.1N (groups 12 & 13) combinations. Grafts were explanted 4 and 8 weeks after transplantation.

Since the immunological responder-status of the host might be involved in determining the kinetics and severity of TA, BN allografts were also transplanted into F344 hosts (group 14). The F344 rat strain displays a similar immunologic responder-status as BN rats, but possesses the Lew MHC-haplotype.

To analyze the contribution of the bone marrow (BM) compartment in determining the severity of TA, MHC-incompatible bone marrow (BM) chimeras were generated (as described below). Six weeks after BM transplantation, DA aortic allografts were transplanted in Lew BM chimeric BN rats (group 15) as well as BN BM chimeric Lew rats (group 16) (Table 1C). Grafts were explanted 12
Transplantations were performed using a fully MHC incompatible third party donor (DA) (Table 1D). DA aortic allografts were transplanted in BN, Lew, BN.1L and Lew.1N recipients (groups 17, 18, 19, & 20, respectively) and were explanted 4 weeks after transplantation.

Table 1. Groups used for aorta transplantation to study the effect of recipient genotype on the development of transplant arteriosclerosis.

<table>
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<th>Group</th>
<th>N</th>
<th>Graft donor</th>
<th>Graft recipient</th>
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<th>non-MHC disparity</th>
<th>Sacrifice (weeks)</th>
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<td>Lew.1N</td>
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</table>

a number of animals analyzed
b weeks after aorta transplantation
c 1 out of 4 animals displayed complete bone marrow chimerism which was used for subsequent DA aortic allografting
d aortic allografting was performed 6 weeks after bone marrow transplantation
Quantitation of transplant arteriosclerosis

Grafts removed at autopsy were fixed in Bouin’s fixative and embedded in paraffin. Tissue sections (7 µm) were taken from the center of each graft and were stained with Lawson’s solution (Klinpath, Duiven, The Netherlands) to visualize elastin fibers. TA was quantitated using a computerized morphometric analysis system (QWin Software, Leica Microsystems B.V., Rijswijk, The Netherlands). Degree of TA was expressed as the total neointimal cross-sectional area (expressed in µm²).

Generation of bone marrow chimeric rats

Both femora and tibiae of the donor rats were excised and surrounding muscle and connective tissue were removed. Both ends were removed with small pincers, and the marrow cavity was flushed with RPMI 1640 (Life Technologies) using a syringe with a needle. Clusters were disaggregated by vigorous pipetting and the cell suspension was filtered using nylon gauze. BM cells were washed, counted and set to a concentration of 2x10⁸ nucleated cells/ml. Male recipient rats, aged 7-10 weeks, were lethally γ-irradiated (BN 8.5 Gy and Lew 9.5 Gy) using a ¹³⁷Cesium source (IBL 673, CIS Bio International). Irradiated BN and Lew hosts received 1x10⁸ Lew and BN BM cells respectively by tail vein injection. Four to six weeks after bone marrow transplantation, chimerism was determined in the peripheral blood by flowcytometry. If complete chimerism (>98% donor cell derived) was observed aortic transplantation of DA allografts into the chimeric hosts was performed.

Statistical analysis

All data are expressed as mean ± SEM. To analyze differences in the degree of neointima formation for statistical significance, the One-Way ANOVA was performed. If this test revealed a significant difference, a Bonferroni’s Multiple Comparison test was performed to analyze differences between subgroups. Differences were considered to be statistically significant when P-values were <0.05.

Results

Kinetics and severity of TA in Lew and BN recipients

First, we analyzed the kinetics and severity of TA in MHC- and non-MHC incompatible aortic allografts transplanted between BN and Lew recipient rats. As shown in Figure 1, 4 weeks after transplantation BN allografts transplanted in Lew hosts showed only weak TA and a subsequent increase with time of the degree of TA was observed reaching a maximum by 24 weeks after transplantation. In contrast, Lew allografts transplanted in BN hosts displayed maximal TA already 4 weeks after transplantation and the magnitude of the TA lesions remained virtually the same over time. Compared to Lew hosts, BN hosts showed significantly more TA 4 and 8 weeks after transplantation (P<0.001 and P<0.05, respectively), whereas at later time points significant differences between the degree of TA in BN and Lew hosts were no longer observed. BN isografts showed only very little TA 12 weeks after transplantation, indicating that the development of TA under these experimental conditions is primarily an allo-immune mediated process.
The rate of development of transplant arteriosclerosis

Severity of TA in MHC congenic Lew.1N and BN.1L recipients

From the data depicted in Figure 1 we defined BN hosts as high TA responders and Lew hosts as low TA responders. Subsequently, we analyzed whether this difference between BN and Lew rats is primarily determined by recipient MHC or non-MHC encoded determinants. Therefore, BN and Lew allografts were transplanted in MHC-congenic Lew.1N and BN.1L hosts, respectively, and the degree of TA present at 4 and 8 weeks after transplantation was compared to the degree of TA present in the (non-congenic) Lew and BN counterparts. Figure 2A shows that allografts transplanted in the Lew to Lew.1N combination show significantly less TA compared to Lew allografts transplanted in BN hosts at both 4 and 8 weeks after transplantation (P<0.001).

On the other hand, as shown in Figure 2B, allografts transplanted in the BN to BN.1L combination showed a tendency of in-
increased TA development compared to the BN to Lew combination at 4 and 8 weeks post transplantation. So, BN.1L hosts tend to develop more TA when compared to the Lew hosts transplanted with a fully MHC disparate BN allograft. These data indicate that the severity of TA is primarily determined by the recipient’s non-MHC haplotype, and that recipients bearing the BN-encoded non-MHC (BN and BN.1L) are high TA responders, whereas recipients bearing the Lew-encoded non-MHC (Lew and Lew.1N) are low TA responders.

Severity of TA in F344 hosts
BN-encoded non-MHC determinants thus seem to predispose for the development of more severe TA, but the precise determinants responsible for this observation are unknown. One possible explanation might be the difference in capacity to produce Th1- and Th2-type cytokines upon stimulation. Lew rats are Th1-prone rats, whereas BN rats rather respond with a Th2-type cytokine response pattern upon stimulation. To analyze whether severity of TA is correlated with a Th2-skewed immune response, aortic transplantation was performed in the fully MHC disparate BN to F344 combination. Like BN rats, F344 rats also show a Th2-skewed immune response, whereas they bear the Lew-derived MHC haplotype. The degree of TA in the BN to F344 strain combination was compared to the degree of TA in the BN to Lew and Lew to BN strain combinations. As shown in Figure 3, the F344 hosts developed moderate TA, similar to allografts transplanted in the BN to Lew combination, indicating that F344 rats are also low TA responders. These results suggest that apparently non-MHC encoded factors other than recipient’s Th2-skewed immune response are involved in determining the rate of development of TA.

Development of TA in Lew bone marrow chimeric BN hosts
The finding, that the T cell responding profile of the recipient is not a major factor in determining the kinetics of TA development was further strengthened by the data of TA development in BM chimeric rats. Lew BM chimeric BN (Lew\textsubscript{BM} ->BN) and BN BM chimeric Lew (BN\textsubscript{BM} ->Lew) rats were generated (as described in the Materials & Methods section) and the level of chimerism was determined by MHC class I haplotype specific flow cytometry. All four Lew\textsubscript{BM} ->BN rats showed complete chimerism, whereas only 1 out of 4 BN\textsubscript{BM} ->Lew rats showed complete chimerism (not shown). Only the complete BM-chimeric rats were transplanted with a DA aortic allograft 6 weeks af-
The rate of development of transplant arteriosclerosis

ter BM transplantation. Ten weeks after aortic transplantation, the Lew\textsubscript{BM}$\rightarrow$BN rats showed definite TA ($0.99\times10^5 \pm 2.34\times10^4 \text{ µm}^2$), whereas in the BN\textsubscript{BM}$\rightarrow$Lew rat no TA could be observed. Although severe perivasculitis and endothelial cell damage indicative of alloreactivity was present (not shown), the eventual development of TA 10 weeks after transplantation seemed to be less pronounced compared to the non-BM chimeric counterparts. However, in the Lew\textsubscript{BM}$\rightarrow$BN rats TA was observed, whereas in the BN\textsubscript{BM}$\rightarrow$Lew rat no TA was detectable, indicating that the BN hosts still possessed the ‘high’ TA responder phenotype, despite complete reconstitution with the Lew-derived lymphoid system.

**Severity of TA in third party (DA) allografts**

Although recipients carrying the non-MHC genotype of the BN strain always displayed high TA responsiveness, even after transplantation of only non-MHC mismatched grafts, transplantation of fully (MHC- and non-MHC) incompatible DA allografts to Lew and BN hosts did not result in the expected TA response patterns. At 4 weeks after transplantation both BN and Lew rats displayed a virtually identical, minimal degree of TA (Figure 4). This was not due to a lack of antigenicity of the DA-grafts, since in the DA to BN.1L combination again, as expected, a strong TA response upon transplantation was observed (Figure 4). These data indicate, that although BN-encoded non-MHC determinants predispose for the rate of development of severe TA, also the specific combination of donor and recipient can significantly influence the overall outcome of this process.

**Discussion**

The development of chronic transplant dysfunction (CTD) and its sequelae remain a major complication in the long-term survival of solid organ transplants. The common histomorphological feature of CTD is development of transplant arteriosclerosis (TA), which is characterized by concentric myointimal proliferation of predominantly vascular smooth muscle cells resulting in the development of an occlusive neointima in the arterial structures of the graft\textsuperscript{1,2,12}. Pathogenesis of TA seems to be multifactorial and risk factors appear to include ischemia/ reperfusion injury, donor brain death, histoincompatibility, acute rejection and viral infections\textsuperscript{1,5}.

Among transplant recipients, however, transplanted with similar HLA-incompatible grafts, receiving similar immunosuppression and exposed to the same known risk factors, variation exists in both the
rejection rate and long-term outcome. This variation has not been fully explained yet, but data indicate that different individuals might display differences in their immune responses against an allograft. These differences in immunologic responder status might at least be in part due to genetic variance in the regulation of cytokine gene expression, which have been shown to correlate with the development of CTD and TA.

In this study we dissected the influence of host MHC- and non-MHC encoded determinants on the development of TA using an aortic transplant model in rats. First, we analyzed the onset and severity of TA in aortic allografts transplanted between Lew and BN hosts. In contrast to Lew hosts, which gradually developed TA with time, BN hosts developed maximal TA already 4 weeks after transplantation (vs. 24 weeks in Lew hosts). The severity of TA in both strains was eventually similar, indicating that rather the rate of TA development than the severity per se was different. Similar observations were reported by Geerling et al. who further showed increased intragraft cytokine mRNA expression levels in BN hosts compared to Lew hosts 1 month after transplantation. Aortic transplantation in MHC-congenic Lew.1N and BN.1L rats revealed that high TA responsiveness is at least in part determined by non-MHC encoded determinants. However, it should be noted that also the interaction between host and graft can influence the severity and kinetics of TA development in certain donor-recipient combinations.

From an immunological point of view, a major difference between Lew and BN rats is the (non-MHC encoded) immunologic responder status and, as a result, the susceptibility for inducible autoimmune diseases. Lew rats display a Th1-skewed immune status and are susceptible for Th1-mediated autoimmune diseases like EAE. In contrast, BN rats display a more Th2-skewed immune profile and are therefore susceptible for Th2-mediated autoimmune diseases like mercuric-chloride induced glomerulonephritis. A Th2-type immune response has been suggested to be associated with the development of TA in rats. We analyzed therefore whether also in our aortic transplant model the high TA responsiveness observed in BN rats was related to the Th2-skewed immune status. However, F344 hosts, which like BN rats display a Th2-skewed responder status, turned out to be low TA responders. These results indicate that although recipient’s non-MHC encoded determinants predispose for low or high TA responsiveness, it is unlikely that the Th1 vs. Th2 immune status of the hosts contributes significantly to this phenomenon. This conclusion is further supported by the notion that also Lew bone marrow chimeric BN rats, in which the original BN lymphoid system has been completely replaced by immune cells derived from the inoculated Lew bone marrow, thereby also changing the immunologic responder profile from a BN to a Lew type, still develop more TA compared to the reciprocal BN bone marrow chimeric Lew rat. Thus, the results presented in this study suggest that the rate of TA development in different rat strains is genetically controlled by non-MHC encoded factors other than the recipient’s immunologic responder status. In a recent study by Harmon et al. it was demonstrated that also in a mouse model of arterial remodeling the mechanism leading to lu-
men narrowing in the vascular remodeling process is genetically controlled, and substantial differences between the different mouse strains were observed\textsuperscript{15}. Current thinking on the process of TA holds that donor-derived medial VSM cells of affected arteries start to proliferate and migrate from the media to the subendothelial space in response to cytokines and growth factors\textsuperscript{3,16,17}. However, we recently demonstrated in our aortic transplant model that the neointimal VSM cells and EC’s are not donor but host-derived. Moreover, recent data indicate that the neointimal VSM cells\textsuperscript{18,19} as well as endothelial cells (EC’s) (J.L. Hillebrands, submitted) are predominantly non-bone marrow derived. Since neointimal lesions predominantly consist of such host-derived VSM cells and EC’s, it is tempting to speculate that these neointimal VSM cells and EC’s intrinsically determine the rate and severity of neointima formation. Which EC and VSM cell produced factors are essential in this process is unknown, but one of the possible candidates is elastase, a VSM cell and EC produced factor involved in the development of TA\textsuperscript{20}. Focal breaks in the internal elastic lamina are a key feature of TA which may be attributed to increased elastase activity\textsuperscript{1,21}. Besides its elastolytic effect, elastases are known to regulate cytokine and growth factor activity (a.o. bFGF and TGF-\(\beta\)), and may, in this way be involved in the development of TA\textsuperscript{20}. Of the rats strains analyzed in this study, BN rats displayed the high TA responder phenotype. Specifically this rat strain shows increased elastase activity, which might be involved in the spontaneous development of elastic laminae defects in BN rats\textsuperscript{22}. However, also other genetic characteristics of BN rats may influence the development of such lesions in elastic laminae\textsuperscript{23}. Inhibition of elastase activity has been shown to attenuate the development of TA in rabbits, indicating that elastase indeed is involved in the process of TA development\textsuperscript{20}.

Also differences in the production of vasoactive peptides between the different rat strains may have contributed to the observed differences in TA responsiveness. For example endothelin-1, an EC produced potent vasoconstrictor, that is mitogenic for VSM cells, has been associated with the development of TA in rats\textsuperscript{24,25}. To our knowledge, however, no data are available on the expression levels of endothelin-1 in different rats strains. Finally, in rats the proliferative response of neointimal VSM cells might also be strain-dependent, as has been demonstrated in a mouse model of arterial remodeling\textsuperscript{15}.

In conclusion, this study shows that the rate of TA development is genetically determined by host non-MHC encoded determinants. In our rat model the BN non-MHC predisposes for high TA responsiveness. However, which gene product(s) precisely determine the rate of TA development remains speculative. Since it is likely that also in the human situation genetic differences between organ transplant recipients will determine the rate of TA development and the long-term outcome of transplantation, identification of the gene products responsible for this phenomenon will be essential both in tracing patients who are at risk to rapidly develop TA, as well as in developing new strategies to prevent or treat TA.
Chapter 5

References


