Chapter 9

General Discussion

Introduction
During the last decades, solid organ transplantation has increasingly become a successful therapy for patients suffering from end-stage organ failure, which is primarily due to the introduction of new immunosuppressive agents preventing acute rejection of the graft. To date, however, clinical transplantation has not achieved its goals as a long-term treatment, and development of chronic transplant dysfunction (CTD) is now recognized as the most important cause of graft loss after the first year after transplantation. CTD can be defined as the progressive irreversible loss of graft function that occurs late in the post transplant period. 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 (VSM) cells intermingled with some T cells and macrophages. Pathogenesis of TA seems to be multifactorial, and both alloantigen-dependent (e.g., acute rejection and histoincompatibility) as well as alloantigen-independent factors (e.g., donor brain death, ischemia and cytomegalovirus infection) have been identified as probable risk factors for the development of TA. The etiology of TA, however, is largely unknown and precise mechanisms involved in the development of this remodeling process still remain obscure.

The aim of this thesis was to analyze the contribution of several risk factors (alloreactivity, CMV infection and host genetics) on the pathogenesis of TA, as well as to gain more insight in the precise mechanism leading to vascular narrowing (origin of neointimal cells). The data described in this thesis have been summarized in a scheme (Figure 1, see spread out figure on page 182) in which a sequence of events is proposed before (I), during (II) and after (III) solid organ transplantation that lead to the development of TA and subsequent CTD.

A central element in the development of TA is injury of the graft vascular tree. As described in detail in Chapter 1 (General Introduction), damage of the graft vasculature may already occur even before graft retrieval. Especially donor brain death and ischemia after graft retrieval, are the main causes of EC activation thereby creating a proinflammatory milieu in the graft during the pre-transplant period (see Figure 1, I). During the actual transplantation, graft endothelium is further activated and may loose function mainly as a result of reperfusion injury to the graft (see Figure 1, II).

In this thesis, we focused on vascular damage inflicted after transplantation, i.e. injury of the graft vasculature as a result of an alloreactive response of the host against the graft, and several factors influencing this process (see Figure 1, III). In the next part of this chapter, the key events leading to TA and CTD will be highlighted in sequence of appearance (as shown in Figure 1) and will be placed in the context of the experimental data described in this thesis. Moreover, possible targets of therapeutic intervention will be discussed.

Alloreactivity and transplant arteriosclerosis (III.a)
Although alloantigen-independent factors may induce damage to the graft before and after transplantation, it has become clear that alloreactivity is the main cause of TA development. Since alloreactivity seems
to be the key player in the development of TA, deletion of the alloreactive response would be a possible strategy to prevent development of TA. Previously, we and others showed that intrathyMIC (IT) immune modulation is effective in preventing acute rejection of allografts in rodents. Though preventing acute rejection and prolonging graft survival, IT immune modulation did not prevent the development of TA in allogeneic cardiac allografts (*Chapter 2*). TA started to develop several weeks after transplantation and an increase in the number of arteriosclerotic coronary arteries as well as in the severity of the neointimal lesions was observed. These histologic changes were associated with an altered intragraft immune response. IT immune modulation did not completely abolish alloreactivity against the graft, and the observed intragraft cytokine profile suggested the presence of Th3 or Tr1 cells in long-term surviving grafts with severe TA. Most tolerance inducing protocols, like our IT immune modulation protocol, mainly partly suppress or alter the alloreactive response (thereby preventing acute rejection), and induction of true tolerance with complete absence of alloreactivity is seldom achieved. Probably all tolerance inducing protocols will fail in preventing TA when alloreactivity is not completely abolished. Most likely, only those protocols will be successful in preventing TA development in which true tolerance is achieved by e.g. donor-specific bone marrow transplantation. Future studies on tolerance induction should therefore aim at developing protocols that induce true tolerance rather than alter the alloreactive immune response. That complete suppression of alloreactivity for a prolonged period of time may indeed be effective in prevention of TA was clear from data described in *Chapter 6*. Daily treatment with high dosages of cyclosporin A completely prevented development of TA in aortic allografts. Long-term treatment using high dosages of currently available immunosuppressive agents, however, is not applicable to clinical transplantation because of serious (nephrotoxic) side-effects.

One of the alloantigen-independent risk factors suggested to be involved in the development of TA is cytomegalovirus (CMV) infection. Although positive correlations between CMV infection and TA development have been shown in clinical transplantation as well as experimental transplant models in rats, also data have been described suggesting that CMV does not contribute to TA development. The mechanism by which CMV could be involved in the pathogenesis of TA is unclear. In *Chapter 4* we showed that infection with Rat CMV indeed can enhance the development of TA in aortic allografts, however, this effect seems to be rather the exception than the rule after allogeneic aorta transplantation. Timing of viral infection appeared to be an important factor in whether or not CMV will enhance the development of TA. Only infection during the developing acute rejection phase resulted in enhanced TA, suggesting that CMV strengthens and prolongs the alloreactive response, thereby increasing vascular damage in the graft. However, CMV-enhanced TA was only observed in one specific weak TA responder rat strain combination, whereas in other weak combinations this effect was not present. These results suggest that al-
though CMV can enhance the development of TA, this effect is dependent on host characteristics influencing TA which may be genetically determined (Chapter 5).

Endothelial cell replacement and transplant arteriosclerosis (III.b)
Several decades ago it was postulated by Woodruff and Medawar\textsuperscript{14,15} that replacement of graft endothelium by host-derived EC’s (‘graft adaptation’) would be beneficial for long-term graft survival. In Chapter 6 we showed that graft endothelium in non-immunosuppressed aortic allografts is indeed replaced by host-derived EC’s. Previously, it has been shown in this model that graft endothelium disappears within 2 weeks after transplantation\textsuperscript{16}, and we now showed complete replacement with host-derived EC’s in this model. In contrast to aortic allografts, in IT immune modulated cardiac allograft recipients, in which alloreactivity was decreased though not completely abolished, graft endothelium was preserved. These results indicate that whether or not graft endothelium will be replaced by host-derived EC’s depends on the severity of graft vascular damage. This was recently confirmed by a study from Lagaaij et al. demonstrating that the level of EC replacement in human kidney allografts is correlated with the severity of vascular rejection\textsuperscript{17}.

Since EC replacement of graft endothelium with host-derived EC’s may occur upon vascular injury, it is of interest to know what the anatomical origin of these EC’s is. In Chapter 8 we showed that the host-derived EC’s in advanced neointimal lesions in aortic allografts are primarily non-bone marrow derived, indicating that a non-marrow source provides these cells. Although migration of EC’s from the recipient side of anastomosis can not be excluded, we propose that a pool of circulating endothelial cells (CEC’s) is the main source of the neointimal host-derived EC’s. It is unlikely that EC replacement will improve long-term graft survival as suggested by Woodruff and Medawar\textsuperscript{14,15}, since both in aortic allografts (EC replacement) as well as cardiac allografts (no EC replacement) development of severe TA was observed (Chapter 2 and Chapter 6). Consequently, it appears that replacement of graft endothelium with host-derived cells is an indicator for severe vascular damage but is not a prerequisite for the subsequent development of TA per se.

Vessel wall morphology and transplant arteriosclerosis (III.c)
Transplant arteriosclerosis can be observed in all intragraft arteries and arterioles, whereas capillaries and veins seem to be protected from these lesions\textsuperscript{5}. Arteries and arterioles consist of a three layer structure: tunica adventitia (fibrous tissue), tunica media (elastin laminae and VSM cells) and the tunica intima (subendothelial fibrous tissue and EC’s). The presence of a pronounced vascular media in arteries and arterioles, which plays an important role in regulating blood flow and blood pressure, appears to be a predominant factor in determining whether vascular structures are prone or resistant to the development of TA. In Chapter 3 we showed that cardiac tissue transplanted subcutaneously following IT immune modulation was characterized by the appearance of graft-derived large veins (neangiogenesis). Although persistent allore-
activity against graft parenchyma and graft EC’s was present, development of TA in these newly formed veins was not observed. These results suggest that only vessels with a medial architecture consisting of elastin laminae and VSM cells are prone to TA development (like coronary arteries in cardiac allografts (Chapter 2) and aortic allografts (Chapter 4 and Chapter 6)), whereas veins and capillaries without a clear medial architecture are resistant to TA. Also in vascularized cardiac allografts neo-angiogenesis with presumably graft-derived veins was observed, which were free of TA, whereas the coronary arteries and arterioles suffered from severe TA (Chapter 2). Since neo-angiogenesis with vascular structures which remain free of TA occurs in transplanted allografts, this might be a possible site of therapeutic intervention to treat CTD with established TA. However, these newly formed blood vessels should be capable of taking over function of the obliterated vessels which is unlikely.

**Response-to-injury hypothesis revisited (III.d)**

Despite discrepancies in histopathology between ordinary atherosclerosis and TA, the ‘response-to-injury’ hypothesis applicable to atherosclerosis and originally proposed by Ross et al.\(^{18,19}\) has been accepted widely for the development of TA. This hypothesis implies that in response to transplant-related trauma (e.g., brain death, ischemia/reperfusion, alloreactivity), which induces EC damage, a generalized vascular repair process is initiated resulting in replication and migration of medial VSM cells into the subendothelial space (schematically represented in Figure 2, Chapter 1). According to this hypothesis, the neointimal VSM cells are of graft-origin and therefore donor-derived. In Chapter 6 and Chapter 7, however, we showed that the neointimal VSM cells both in aortic and cardiac allografts are not of donor but of host-origin. Our findings have recently been confirmed by others also showing contribution of host-derived VSM cells in the process of TA development in femoral artery allografts\(^{20}\) in rats and aortic allografts in mice\(^{21-23}\). Moreover we showed that these host-derived VSM cells are predominantly, if not all, derived from a non-bone marrow source (Chapter 8). This latter observation is in line with some recent observations in aortic transplant models in mice demonstrating that, though bone marrow-derived VSM cells contribute to the development of TA, the percentage of these cells is rather low\(^{22,23}\). Although derived from a non-marrow source, the precise anatomical origin of the host-derived neointimal VSM cells is as yet unknown. At least two possibilities arise from which anatomical origin the VSM cells are derived: ingrowth from the recipient side of anastomosis or recruitment of VSM (progenitor) cells from the circulation. Although the first possibility may take place, our data suggest that the bulk of VSM cells are blood-borne since a scattered distribution of \(\alpha\)-actin positive cells adhering to the developing neointima in aortic allografts was observed (Figure 2). Taken together, TA indeed seems to be a response-to-injury, however, not VSM cells and EC’s from the injured graft but host-derived cells are the key players in this remodeling process.

The data described in Chapter 6, Chapter 7 and Chapter 8 have led to the hypo-
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Theoretical sequence of events in the aortic and coronary artery vascular wall leading to TA as schematically depicted in Figure 3. The process of TA development can be divided in 5 phases, starting from the transplantation procedure (phase 1) to end-stage TA (phase 5) and will be discussed below. Although this scheme is based on our observations in the aortic and cardiac transplant models in the rat, this sequence of events may also apply to solid organ transplants in clinical transplantation.

**Phase 1.** At the time of transplantation an anastomosis (e) is made between recipient and donor blood vessels both having a regular 3-layer microscopic anatomy consisting of a tunica adventitia (a), tunica media (b) and tunica intima (c). The vasa vasorum (d) provide nutrition to the outer part of the vascular wall. VSM cells are located between the elastic laminae in the media. At the luminal side the vessel wall is covered with EC’s. At this early time point after transplantation, the graft endothelium may already be activated or damaged as a result donor brain death, ischemia and reperfusion.

**Phase 2.** The early phase of tissue damage after transplantation is primarily due to host alloreactivity directed against the graft. This is characterized by ingrowth of recipient derived vasa vasorum in the graft adventitia (a) and extensive perivasculitis around these small vessels (b). Moreover, graft endothelium is attacked by recipient inflammatory cells leading to destruction of the EC-lining of the graft (c). As a result of this alloreactive response, medial VSM cells start to disappear as a result of apoptosis (d).
**Phase 3.** Due to the ongoing inflammation in the adventitia (a) and/or the lack of ingrowth of new recipient derived vasa vasorum into the media (thereby causing a severe nutritional deficiency for that area) complete necrosis disappearance of the medial VSM cells is observed, with only the elastin scaffold remaining (b).

**Phase 4.** Rebuilding of the damaged vessel wall, which has lost appropriate function, starts: a new EC-layer of recipient origin (re-endothelialization) is built both by ingrowth of recipient EC’s over the denuded intima starting at the side of anastomosis (a) as well as derived from circulating EC’s which may originate from a non-bone marrow source such as the host vascular wall (b & c).

**Phase 5.** Like for EC recovery, VSM cell replacement starts either through ingrowth of recipient VSM cells into the graft intimal space starting at the site of anastomosis (a), or from circulating recipient VSM (progenitor) cells (b). Initial appearance of VSM cells is followed by intimal hyperplasia caused by uncontrolled proliferation of VSM cells, resulting in luminal occlusion eventually (c).

**Genetic predisposition and transplant arteriosclerosis (III.e)**

Besides the aforementioned alloantigen dependent and independent factors involved in TA, other host determined genetic factors may modulate the rate and severity of the development of TA\(^{24}\). In the literature, several data have been reported indicating that development of TA is influenced by genetic variation in the regulation of cytokine gene expression, in particular variation in the expression levels of TGF-β\(^{25-27}\).

In Chapter 5 we showed that besides genetic differences in the immunological responder status of the host also other non-MHC encoded determinants influence the rate of development of TA in aortic allografts. These results indicate that the rate of TA development is indeed genetically controlled, however which factors are responsible for this effect remains unclear. Since neointimal lesions predominantly consist of host-derived VSM cells and EC’s (Chapter 6 and Chapter 7), it is tempting to speculate that these neointimal VSM cells and EC’s intrinsically determine the rate and severity of neointima formation. Possible candidates for VSM cell or EC produced factors which may be involved in the development of TA are enzymes (e.g., elastase\(^{28}\)) and vasoactive peptides (e.g., endothelin-1\(^{29}\)). Also the proliferative capacity of VSM cells may be genetically determined \(^{30}\), and differences in proliferative capacity may influence the rate and severity of TA development.

Since genetic differences between organ transplant recipients may 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 (genetically predisposed) to develop TA rapidly, as well as in developing new strategies to prevent or treat TA.

**Transplant arteriosclerosis: a normal vascular repair process beyond control?**

We propose that the development of TA is a repair process initiated from the host attempting to restore vascular wall function of vessels in which the medial architecture has been destroyed by an alloreactive inflammatory response\(^{31}\). The ques-
Phase 1.

Normal vessel wall morphology at the time of transplantation consisting of the adventitia (a), media (b), intima (c) and vasa vasorum (d). Anastomosis (e).

Phase 2.

Ingrowth of recipient-derived vasa vasorum (a). Alloreactive response directed against advential tissue (b) and EC's (c) results in medial VSM cell apoptosis (d).

Phase 3.

Ongoing inflammation (a) results in complete disappearance of medial VSM cells (b) and graft EC's.

Phase 4.

Re-endothelialization with host-EC's via ingrowth from the side of anastomosis (a) and/or from a pool of CEC's (b & c).

Phase 5.

Recruitment of host-derived VSM cells via ingrowth from the side of anastomosis (a) and/or from the circulation (b), resulting in intimal thickening (c).

Figure 3. Schematic representation of events in the vascular wall after allogeneic organ transplantation leading to transplant arteriosclerosis. The different phases are described in detail in the text. Abbreviations: CEC, circulating endothelial cell; EC, endothelial cell; IC, inflammatory cell; VSM cell, vascular smooth muscle cell.
tion remains why a healing process, that normally is self-limiting once function is fully restored, in TA does not stop and eventually will lead to occlusion of the vessel lumen. One explanation is that as a result of the ongoing perivascular inflammatory response, activation and damage of vascular wall cells persist and mitogenic factors like PDGF and bFGF will be produced continuously. Alternatively, physiologic shortcomings of the neointima to restore vascular wall function may be the driving force behind the ongoing intimal thickening observed in TA. This explanation is founded by observations made in biodegradable vascular grafts, which have been developed to replace diseased vessels (e.g., in cardiac coronary surgery). Experiments in rats have shown that these grafts should be biocompatible, microporous, compliant, and biodegradable. Essentially, the graft functions as a temporary scaffold allowing de novo formation of a new vessel wall in parallel with the biodegradation process. With time a neointima and neomedia were found to develop inside the lumen of the graft. The process leading to TA may essentially be the same as the process observed in the biodegradable compliant vascular grafts: as a results of the rejection process medial VSM cells are destroyed leaving the now condensed layers of elastic laminae as a scaffold facilitating regeneration of a new vessel wall. Major difference between TA and biodegradable vascular grafts is that in the latter within the neomedia new elastic laminae developed with circularly oriented VSM cells. In TA, however, neointimal VSM cells are randomly oriented and newly formed elastin is hardly present. Since a well organized neomedia in biodegradable vascular grafts may restore graft function, the random organization of neointimal VSM cells without elastic laminae in TA does not.

Possible targets for therapeutic intervention to prevent of treat transplant arteriosclerosis

Based on the observations described in this thesis several possible targets of therapeutic intervention could be identified, which may be helpful in developing new strategies to prevent or treat TA in the future. To our opinion, most profit can be made by strategies which are directed on prevention of TA rather than treatment of established TA. The key events leading to TA which can be used as a point of application are shown in gray boxes in Figure 1 and will be discussed below.

Suppression of the alloreactive response

Damage induced by the alloreactive response appeared to be the most important factor for TA development. Therefore, deletion of host’s alloreactivity against the graft should be the obvious solution to prevent TA after organ transplantation. New immunosuppressive drugs which abolish any degree of alloreactivity but have not the toxic side-effects of today’s immunosuppressive agents may be effective in preventing TA. Also tolerance induction protocols which induce true tolerance rather than alter or diminish alloreactivity (Chapter 2) should be effective in preventing allo-mediated vascular damage, thereby also preventing the development of TA. True tolerance might be achieved by donor-specific bone marrow transplantation, and experimental data in rodents indicate that bone marrow transplantation indeed can prevent develop-
ment of TA\textsuperscript{36,37}. However, bone marrow transplantation is still a risky procedure, particularly when the transplantation is performed across one or more MHC incompatibilities, and today these risks do not counterbalance the risk of TA development after organ transplantation.

\textit{Anti-cytomegalovirus prophylaxis}

CMV-infection may enhance the development of TA in susceptible patients, in particular when active CMV infection coincides with acute rejection episodes (\textit{Chapter 4}). Active CMV infection should therefore be prevented and this can be achieved by prophylaxis with anti-viral agents (e.g., ganciclovir). Today, some transplantation-centers already incorporate anti-CMV prophylaxis in their standard treatment regimens. Although anti-CMV prophylaxis by itself will not prevent development of TA, it may be effective in reducing the rate and severity of TA development.

\textit{Prevention of medial VSM cell apoptosis and uncontrolled neointimal proliferation}

We proposed that losing vascular wall function as a result of disappearance of medial VSM cells is the key element in inducing TA. Preservation of the vascular media should retain vascular wall function, thereby preventing development of TA. Recently, it has been shown that medial VSM cells disappear as a result of apoptosis. Therapies directed at prevention of medial VSM cell apoptosis despite the presence of persisting alloreactivity might therefore be effective in the prevention of TA.

Once the medial VSM cells have disappeared and host-derived VSM cells have been recruited to form the neointima, a possible way of intervention is prevention of VSM cell proliferation beyond the stage of functional cell repair. However, to do so we first need to identify the precise (possibly autocrine) factors which regulate proliferation of neointimal VSM cells.

\textit{Identification of high-risk patients}

Since genetic differences between organ transplant recipients will determine the rate of TA development, identification of the gene products, possibly produced by host-derived VSM cells, responsible for this phenomenon will be essential in tracing patients who are genetically predisposed to develop TA rapidly and may provide new tools for therapeutic intervention. Immunosuppressive regimens could be adjusted for such high TA responder patients.

\textit{Concluding remarks}

In this thesis we have identified the contribution of several risk factors in the pathogenesis of TA. Moreover, we acquired a better understanding in the precise mechanisms leading to vascular narrowing, and showed that in contrast to the current dogma, neointimal EC’s and VSM cells are not of donor-origin but of host-origin. Putting the data described in this thesis together, we propose that instead of looking at TA as an undesirable element in chronic transplant dysfunction, the process leading to TA is basically part of a normal healing process and as such should be welcomed. However, this “healing process”, which may be genetically controlled, does not seem to stop, eventually leading to total occlusion of the vessels involved. This revised view on the development of TA has led to the identification of possible new sites for thera-
peutic intervention to prevent TA and provides a basis for future studies in the strug-gle against TA after solid organ transplantation.

References


Figure 1. Scheme of the proposed sequential events before and after solid organ transplantation that lead to the development of transplant arteriosclerosis and chronic transplant dysfunction. Key events addressed in this thesis are highlighted and the chapters in which the regarding data are described are given. Possible targets of therapeutic intervention to prevent transplant arteriosclerosis are shown in gray boxes.