# Chronic transplant dysfunction and transplant arteriosclerosis: new insights into underlying mechanisms

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Although effective in the short-term, clinical solid-organ transplantation has not achieved its goals as a long-term treatment for patients with end-stage organ failure. Development of so-called chronic transplant dysfunction (CTD) is now recognised as the predominant cause of allograft loss long-term (after the first post-operative year) following transplantation. CTD has the remarkable histological feature that the luminal areas of intragraft arteries become obliterated, predominantly with vascular smooth muscle cells intermingled with some inflammatory cells. The development of this transplant vasculopathy, referred to as transplant arteriosclerosis (TA), is a multifactorial process and many risk factors have been identified. However, the precise pathogenetic mechanisms leading to TA are largely unknown and, as a result, current prevention and treatment protocols are inadequate. This review discusses the risk factors for TA and current views on the pathogenetic mechanisms leading to this vasculopathy. We argue here that host-derived cells contribute to the development of these vascular lesions, and propose that TA results from a normal vascular repair process that proceeds beyond the needs of functional repair. Guided by the proposed sequence of events, we finally discuss possible directions for future intervention strategies to prevent TA after solid-organ transplantation.

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Since the late 1950s, transplantation of solid organs has become an increasingly successful therapy for patients suffering from end-stage organ failure. For example, in the USA in the year 2000, >13 000 kidneys, ~5000 livers, >2000 hearts and ~1000 lungs were transplanted (Ref. 1). The short-term results of organ transplantation have significantly improved over recent decades. This improvement is primarily a result of the use of more-effective immunosuppressive agents. In particular, the introduction of cyclosporine A (CsA) in the late 1970s caused an enormous decrease in morbidity and mortality following solid-organ transplantation (Ref. 2). Moreover, improved histocompatibility leukocyte antigen (HLA) tissue-typing assays and surgical techniques, as well as advances in donor-organ preservation, contributed to the decreased morbidity and mortality after solid-organ transplantation.

# Allograft rejection

After transplantation of allogeneic solid organs, grafts can be rejected, resulting in graft loss, in three different ways: hyperacutely, acutely and chronically.

Grafts are hyperacutely rejected if pre-existing circulating anti-donor-HLA antibodies are present in the recipient at the time of transplantation. Binding of these antibodies to HLA antigens expressed on donor endothelium results in severe damage to the graft endothelial cells (ECs), and platelet aggregation and complement activation occur. Today, however, potential transplant recipients are frequently panel-tested for the presence of circulating anti-HLA antibodies before transplantation, thereby minimising the risk of hyperacute rejection.

Unlike hyperacute rejection, which is antibody-mediated, acute rejection is a cellmediated pathological inflammatory response, which usually occurs in the first few months after transplantation (Ref. 3). Acute rejection occurs when HLA disparities between donor and recipient are present. Because of limitations in the time donor organs can be preserved, and the shortage of donor organs, prospective HLAtyping of donors is not possible except for kidney grafts. Therefore, the presence of HLA mismatches between donor and recipient cannot be prevented, thereby increasing the risk of acute rejection. However, in recent years it has been possible to treat acute rejection episodes adequately with immunosuppressive agents such as CsA, FK506 (Tacrolimus) and rapamycin (Sirolimus).

Nevertheless, despite the use of these new drugs, clinical transplantation has not achieved its goals as a long-term treatment. Although a steady improvement in the long-term survival of renal grafts since the late 1980s has been described (Ref. 4), this effect is less clear in other organs, and no new drugs are available to extend graft survival time further. Chronic transplant dysfunction (CTD) is now recognised as the primary cause of allograft loss after the first year following transplantation (Ref. 5).

### Chronic transplant dysfunction

CTD can be defined clinically as the progressive irreversible loss of graft function that occurs late in the post-transplant period (months to years after transplantation) (Ref. 6). Formerly, CTD was also known as 'chronic rejection'. The term 'rejection' implies the alloimmune response of the recipient against the graft is the basis for the deterioration in graft function. However, there is now evidence that alloantigen-independent factors can cause similar functional and histopathological changes after transplantation. Referring to the whole process as chronic rejection is therefore not satisfactory, and as long as the progressive deterioration in graft function cannot exclusively be attributed to an alloimmunemediated pathway it is recommended the process is termed CTD, which avoids implication of a causative factor (Ref. 7).

The incidence of CTD after transplantation depends on the type of organ grafted, and varies from 3% in liver allografts to >70% in lung allografts five years after transplantation (Table 1) (Refs 8, 9). The clinical presentation of CTD generally implies deterioration of graft function; specific clinical parameters for each organ graft are shown in Table 1 (Refs 7, 8).

# **CTD**-associated histopathology

The histopathological characteristics associated with CTD vary between the different organs (Refs 7, 8, 10) (Table 1), but there is a common histomorphological feature of CTD in kidney, heart and lung transplants: transplant vascular sclerosis or transplant arteriosclerosis (TA) (Refs 3, 10, 11). However, although TA can be found in transplanted kidneys, hearts and lungs, the frequency of this vasculopathy within the different organs is not the same, and renal grafts

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# Table 1. Incidence, clinical presentation and histopathology of chronic transplant dysfunction (CTD) after solid-organ transplantation (tab001jhg)

Organ	CTD description	Incidence (after 5 years)	Clinical presentation	Histopathology
Kidney	Chronic rejection	30–50%	Increased glomerular filtration rate; increased plasma creatinine concentrations; proteinuria; arterial hypertension	Tubular atrophy; glomerular sclerosis; inflammation; fibrosis; arteriosclerosis
Heart	Transplantation- associated arteriosclerosis	>50%	Myocardial infarction; arrhythmias; sudden death	Inflammation; fibrosis; arteriosclerosis
Lung	Bronchiolitis obliterans syndrome	>70%	Increased pulmonary function	Obliteration bronchioli; inflammation; fibrosis; arteriosclerosis
Liver	Vanishing bile duct syndrome	3–26%	Increased liver enzymes in blood; increased bilirubin in blood	Vanishing bile ducts; inflammation; fibrosis; arteriosclerosis

can show CTD without TA; thus, TA is not an absolute prerequisite for deteriorated graft function after transplantation (Ref. 12).

TA is characterised by vascular lesions in the graft that consist of concentric myointimal proliferation, resulting in the development of an occlusive neointima in the graft arterial structures (Ref. 6). The neointima primarily comprises  $\alpha$ actin-positive vascular smooth muscle (VSM) cells (Fig. 1), which have been thought to be derived from the vessel media (Ref. 8). This progressive blood-vessel occlusion could lead to downstream ischaemic tissue damage and disruptive fibrosis, and has therefore generally been accepted as the main cause of progressive deterioration in graft function (CTD). Other findings coinciding with TA include a persistent focal perivascular inflammation (perivasculitis), endothelial swelling and inflammation (endothelialitis), disruption of the internal elastic lamina, focal myocyte necrosis, foam cell accumulation in the neointima, and presence of macrophages and T cells in the neointimal lesion (Ref. 13). In contrast to ordinary atherosclerosis, which is usually focal and eccentric, the common form of TA is concentric and generalised.

# Aetiology of CTD

The presence of persistent perivascular inflammation in TA suggests that alloresponse-

mediated injury of the graft vessels is the prime cause of TA development. Indeed, in 1963 Porter et al. had already described TA in kidney allografts from patients who had experienced early episodes of acute rejection (Ref. 14), suggesting that early acute cellular infiltration of the graft (acute rejection) might evolve into a more-chronic inflammatory process. However, the aetiology of TA remains poorly defined. The pathogenesis of TA seems to be multifactorial, and although alloreactivity of the host against the graft appears to be the most important factor contributing to the development of TA, alloantigen-independent factors are also associated with the pathogenesis of TA (Ref. 15).

Risk factors for TA development identified so far can be roughly divided into alloantigendependent factors (i.e. a consequence of the donor-recipient combination), donor-related factors, recipient-related factors, and viral infections.

# Alloantigen-dependent risk factors *Histoincompatibility*

The presence of HLA disparities between donor and recipient is associated with acute rejection of the graft, and long-term graft survival appeared to be strongly correlated with the presence of HLA incompatibility between the donor and the recipient (Refs 16, 17, 18). However, a clear



**Figure 1. The histopathology of transplant arteriosclerosis.** (a) Normal morphology of artery. The artery wall is composed of three layers with distinct cellular and matrix compositions: the adventitia, media and intima. The photomicrograph is a confocal laser scanning microscopic image of a normal, non-transplanted, rat aorta, stained for DNA (DAPI, blue), vascular smooth muscle (VSM) cell  $\alpha$ -actin (green) and endothelial cells (red). Magnification, X100. (b) Transplant arteriosclerosis (TA), characterised by perivascular inflammation, disappearance of medial VSM cells, and formation of a neointima containing VSM cells. The photomicrograph is a confocal laser scanning microscopic image of TA in a rat aortic allograft three months after transplantation, triple stained for DNA (DAPI, blue), VSM cell  $\alpha$ -actin (green) and endothelial cells (red). Magnification, x100 **(fig001jhg)**.

association between the number of HLA mismatches and the development of TA remains a matter of controversy (Refs 18, 19). In experimental transplant models, isografts (having no mismatches) develop no or minor TA after transplantation, suggesting that HLA incompatibility indeed plays a role in the development of TA (Ref. 20). However, it is unclear whether matching of donor and recipient HLA directly affects the development of TA or whether the effect on TA results from a decreased incidence of acute rejection episodes.

# Acute rejection

Although no direct association between the number of HLA mismatches and the development of TA is observed, one of the most important risk factors for the development of TA is the onset, frequency and severity of acute rejection episodes after transplantation. This effect has been demonstrated in many retrospective studies analysing all types of organ transplants (Refs 19, 21, 22). These clinical data have been confirmed by experimental studies using animal transplant models (Ref. 23). However, the occurrence of acute rejection episodes early after transplantation is not an absolute prerequisite for the development of TA, since development of TA without prior acute rejection episodes has also been described (Ref. 24).

### Inadequate immunosuppression

Since acute rejection is associated with TA development, inadequate immunosuppression might also be related to the development of TA. In several clinical studies it indeed has been shown that a low dose of maintenance CsA was associated with CTD (Refs 25, 26). Moreover, studies in rats showed that high-dose CsA treatment prevents or inhibits development of TA after allogeneic aorta transplantation (Ref. 27). Although very effective in rats, in humans it would be impossible to use high doses of CsA for prolonged periods of time because of the nephrotoxic side effects (Ref. 28) (Box 1). Noncompliance of patients might result in inadequate immunosuppression as well, and indeed it has been shown that non-compliance is associated with late deterioration in graft function (Ref. 29).

# Anti-donor-antigen antibodies

Although many patients develop antibodies that are reactive with donor HLA antigens and other

donor-tissue antigens after transplantation, a clear correlation between antibodies and CTD development is not consistently found (Ref. 30). After experimental kidney transplantation in rats, antibodies reactive with glomerular and tubular basement membranes, mesangial cells and ECs were found in sera of allograft recipients (Ref. 31). Donor-reactive antibodies have also been detected after allogeneic aorta transplantation (Ref. 32). The exact role of donor-reactive antibodies in the pathogenesis of TA needs to be further explored.

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# Donor-related risk factors Ischaemia

Whether or not graft ischaemia, which can occur after graft retrieval, plays an important role in the development of CTD and TA is still unclear, since studies implicating (Ref. 16) and discounting (Ref. 33) the effect of ischaemia have been described. In experimental kidney and aorta transplant models in rats, prolonged ischaemic time induced the development of TA in isografts (Ref. 34). Moreover, the severity of the vascular lesions seems to correlate with the duration of the ischaemic period in isografts (Ref. 35), although in allografts this remains controversial.

# Brain death

Kidney allografts from brain-dead donors show decreased long-term graft survival compared with grafts obtained from living related and unrelated donors (Ref. 36). It has been hypothesised that brain death increases the cytokine-induced expression of surface molecules on peripheral organs, and in experimental models brain death indeed activates ECs, increases the influx of inflammatory cells and accelerates the onset of acute rejection (Ref. 37). Whether brain death also accelerates the development of TA has still to be proven.

### Donor age

Kidney and cardiac allografts obtained from older donors are associated with poorer survival rates and show an earlier onset of CTD (Ref. 38).

# **Recipient-related risk factors** *Cytokine gene polymorphism*

Among organ recipients transplanted with similar HLA-incompatible grafts and receiving similar immunosuppression, variation exists in both the rejection rate and long-term outcome.

# Box 1. Current immunosuppressive protocols to prevent chronic transplant dysfunction

The standard therapy to prevent acute rejection in cardiac transplantation is a triple therapy consisting of cyclosporine A (CsA), azathioprine and prednisone (Ref. 126). CsA is a small fungal peptide that interferes with the synthesis of a variety of cytokines, particularly interleukin 2, which is critical for T-cell maturation and clonal expansion (proliferation), and interferon  $\gamma$ , which is critical for macrophage activation (Ref. 127). Azathioprine is a purine antagonist and inhibits cell proliferation (Ref. 128). Prednisone is a corticosteroid and among other effects influences the transcription of several genes, thereby inhibiting T-cell proliferation (Ref. 129). Although this triple therapy is very effective in treatment of acute rejection episodes, CsA therapy has some serious side effects. CsA induces severe nephrotoxicity, and even at minimal effective concentrations of CsA evidence of nephrotoxicity can be present (Ref. 127). Moreover, CsA therapy does not prevent development of chronic transplant dysfunction (CTD), which might be attributed to the fact that CsA directly upregulates fibrogenic cytokines such as transforming growth factor  $\beta$  (Ref. 130).

Over the past decade, several new immunosuppressive drugs have been developed, of which some are now being used in clinical practice. FK506 (Tacrolimus) is more potent than CsA, but has the same mode of action. However, FK506 has not been found to prolong long-term graft survival after kidney transplantation beyond that achieved with CsA (Ref. 131). Mycophenolate mofetil (MMF) and rapamycin (Sirolimus) are more-recently approved immunosuppressive drugs, and both inhibit T-cell proliferation (Refs 132, 133). These new immunosuppressive drugs also have side effects and appear not to prevent or control CTD. Several studies in rats, however, indicate that treatment of allograft recipients with CsA, rapamycin or other immunosuppressive agents can block the development of CTD-related vasculopathy (Ref. 6). Rapamycin is of particular interest since in vitro it inhibits growth-factor-driven proliferation of vascular smooth muscle cells and disrupts signal transduction by a variety of cytokines such as epidermal growth factor and platelet-derived growth factor. Moreover, rapamycin combined with CsA therapy reduces the incidence of acute rejection episodes in humans, allowing a lower CsA dose as a result of synergistic effects (Ref. 118).

In addition to immunosuppressive drugs, polyclonal antibodies specific to T cells, such as antithymocyte globin (ATG) and anti-CD3 (OKT3), are mainly used as rescue therapy; they result in functional T-cell blockade if patients do not respond to regular immunosuppressive drugs.

From an immunological point of view, different individuals might display different responses upon allogeneic (transplanted graft) stimulation. This individual variation is, at least in part, due to genetic variation in the regulation of cytokine gene expression (Ref. 39). High and low cytokine responses in vitro for tumour necrosis factor  $\alpha$ (TNF- $\alpha$ ), transforming growth factor  $\beta$  (TGF- $\beta$ ), interferon  $\gamma$  (IFN- $\gamma$ ) and interleukin 10 (IL-10) can be predicted from an individual's cytokine genotype (Ref. 40). Acute rejection of kidney allografts has been correlated with the high TNF- $\alpha$ , IFN- $\gamma$  and IL-10 production genotypes, although some controversy exists (Refs 41, 42, 43). The high TGF- $\beta$  production genotype correlated with accelerated onset of TA in cardiac allografts (Ref. 44) and increased risk for the development of obliterative bronchiolitis following lung transplantation (Ref. 45).

Genetic polymorphisms in genes outside of the major histocompatibility complex (MHC) have also been associated with the development of TA; for example, genetic variation in the angiotensin-I-converting enzyme (ACE) gene (associated with high ACE production) in both donors and recipients of cardiac allografts seems to be associated with the development of TA (Refs 46, 47).

# Hypertension and hyperlipidaemia

In clinical kidney and heart transplantation, systemic hypertension has been shown to be associated with CTD (Ref. 48). Moreover, in experimental kidney transplantation systemic hypertension accelerates CTD, whereas antihypertensive drugs inhibit or reduce TA and CTD after kidney and aorta transplantation (Ref. 49). Hyperlipidaemia has also been identified as

6

a risk factor for CTD (Ref. 50); however, its role remains controversial.

# Gender and race

Male recipients are more vulnerable to CTD compared with female recipients (Ref. 51). This gender effect might reflect a role for oestrogen, as it has been shown that oestradiol effectively inhibits TA after allogeneic aorta transplantation in rats (Ref. 52). Moreover, long-term graft survival of cardiac and kidney allografts seems to be related to race (Ref. 51).

# Cytomegalovirus

In the late 1980s a positive correlation between the presence of cytomegalovirus (CMV) (positive serology, CMV inclusion bodies, and positive CMV culture) and human cardiac graft atherosclerosis was demonstrated (Ref. 53). CMV has also been shown to be associated with the development of CTD in kidney, liver and lung transplants (Refs 22, 25, 54). In contrast, some data indicate that CMV does not contribute to enhanced CTD after solid-organ transplantation (Ref. 55). These different clinical outcomes might be related to differences in antiviral treatment regimens (e.g. pre-emptive therapy or prophylactic treatment with antiviral drugs).

Several experimental transplant models in rats (heart, kidney, liver and trachea), using rat CMV (RCMV), have also indicated that viral infection accelerates the development of CTD-related pathology (Refs 56, 57). In the aortic transplant model in rats, RCMV infection enhanced both the perivascular inflammatory response (Refs 58, 59) and neointima formation (Refs 60, 61, 62, 63). Inhibition of viral replication using antiviral drugs (Refs 60, 63) and of inflammation using immunosuppressive drugs (Ref. 64) prevents RCMV-enhanced TA development.

# Pathophysiology of TA

Despite discrepancies in histopathology between ordinary atherosclerosis and TA, the 'responseto-injury' paradigm applicable to atherosclerosis and originally proposed by Ross et al. (Ref. 65) has been accepted widely for the development of TA. This paradigm proposes that transplantrelated trauma (alloantigen-dependent and alloantigen-independent) causes activation and damage of ECs along the graft arterial system. Important insults leading to EC damage might include preservation/ischaemia injury, reperfusion injury, acute rejection episodes (i.e. activation of alloreactive T cells), antibody deposition, and complement fixation (Ref. 66). The thus damaged and activated endothelium subsequently initiates a generalised remodelling process that is co-ordinated by proinflammatory and histogenic factors produced by the activated ECs themselves as well as vessel-wall parenchymal cells and inflammatory cells. Moreover, the immune response characterised by perivascular inflammation induces further lowgrade damage to the vascular endothelium. This cascade of events is thought to result eventually in replication of VSM cells in the vascular wall, influx of VSM cells from the media into the subendothelial space (intima), and generation of the neointimal lesion (Ref. 8). So, according to this hypothesis, graft endothelium seems to be one of the key players in the development of TA.

# Role of endothelium in inflammation and T-cell activation

The initial damage to the graft endothelium, induced by alloantigen-independent factors, causes upregulation of adhesion molecule expression [e.g. E-selectin, P-selectin, vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1)]. This subsequently results in increased interactions of polymorphonuclear neutrophils (PMNs) and lymphocytes with ECs (Ref. 67). Moreover, several chemokines produced by both endothelium and interstitial cells [e.g. IL-8, monocyte chemotactic protein 1 (MCP-1), monocyte inflammatory protein  $1\alpha$  (MIP- $1\alpha$ ), MIP- $1\beta$  and RANTES ('regulated by activation normal T-cell expressed and secreted')] can promote the binding of lymphocytes and macrophages to endothelium (Ref. 68). Influx of PMNs is followed by influx of macrophages into the subendothelial space, which might subsequently be activated by IFN-γ. Activated macrophages produce several inflammatory mediators, including IL-1, IL-6, IFN- $\gamma$ , TNF- $\alpha$  and the chemokines IL-8, MCP-1, MIP-1 $\alpha$  and MIP-1 $\beta$ . These cytokines and chemokines in turn further activate the graft endothelium and increase the expression of adhesion molecules, thereby attracting more macrophages and lymphocytes in a positivefeedback loop (Ref. 69). Thus, as a result of the transplant procedure, a complete network of cytokines is already activated, even before the allogeneic reactions develop.

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7

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In addition to its ability to attract and activate macrophages, graft endothelium can activate alloreactive T cells by the direct allorecognition pathway of antigen presentation. In the indirect pathway, nominal antigens are presented as peptides to (self-restricted) T cells by self-MHC molecules, whereas in direct allorecognition a T cell recognises foreign MHC molecules per se independent of the peptides present in their grooves (Ref. 70). In vitro studies have shown that ECs express both MHC class I and II antigens upon stimulation with IFN- $\gamma$ , and are able to directly stimulate allogeneic T cells (Ref. 71). In addition to MHC molecules, ECs also express costimulatory molecules required for full T-cell activation. The best defined of these molecules is LFA-3, which interacts with T-cell CD2 (Ref. 72). Once activated, T cells produce leukotrienes and several cytokines, including IL-1, IL-4, IFN- $\gamma$ , TNF- $\alpha$  and TGF- $\beta$ , which increases the expression of E-selectin, ICAM-1 and VCAM-1 on graft endothelium (Ref. 73). Moreover, TNF- $\alpha$  and IFN- $\gamma$  further increase the expression of MHC class I and II on graft ECs. This cross-talk between lymphocytes and ECs suggests that a positive feedback loop might be established.

# Role of VSM cells in the development of TA

One central element of the TA paradigm is that smooth muscle cells from the vessel media are the progenitors of the neointima - that is, medial VSM cells migrate from the media to the intima in response to activation by cytokines and growth factors (Ref. 8). The ongoing perivascular response induces persistent low-grade damage to the graft endothelium, which in turn begins to secrete growth factors to repair the damage. Upon activation, ECs have been shown to produce multiple growth factors, such as platelet-derived growth factor (PDGF), insulin-like growth factor (IGF-1), basic fibroblast growth factor (bFGF) and TGF- $\beta$ , as well as pro-thrombotic molecules (tissue factor and plasminogen activator inhibitor) and metabolic products such as prostaglandins, nitric oxide and low-density lipoproteins (Refs 8, 65). Activated T cells and macrophages also produce a range of these factors. The 'response-to-injury' paradigm implies that these factors together induce migration of VSM cells to the intima, during which they transform their phenotype from 'contractile' to 'synthetic' and become capable of replication (Ref. 65).

# Donor versus recipient origin of neointimal cells in TA

TA consists of progressive concentric intimal thickening coinciding with ongoing perivascular inflammation. Current thinking on the process of TA, as described above, holds that in response to cytokines, growth factors and other inflammatory mediators produced by inflammatory cells and damaged/activated graft endothelium, donor-derived medial VSM cells of affected arteries start to proliferate and migrate from the media into the subendothelial space just beneath the EC layer (Ref. 10). If the neointimal ECs and VSM cells do indeed originate from the donor, they should be demonstrably graft derived.

As long ago as the early 1960s, Woodruff (Ref. 74) and Medawar (Ref. 75) proposed that replacement of graft endothelium with hostderived ECs might be the reason why long-term allograft survivors experience relatively few rejection episodes (i.e. graft adaptation). Since then, several groups have studied whether ECs covering the luminal surface of the graft vessels after transplantation are indeed of donor origin or have been replaced by host-derived ECs. Recently, the donor versus host origin of VSM cells has also been examined.

# Donor origin

A donor origin of the graft vessel endothelium is supported by findings reported by several groups studying human cardiac, kidney and lung allografts (Refs 76, 77). Only a few studies have addressed the question of whether the neointimal VSM cells in TA are indeed graftderived. A donor origin of neointimal VSM cells has been described in human solid-organ transplants, favouring the current paradigm that neointimal VSM cells are derived from the graft's media (Refs 77, 78).

Neointimal VSM cell proliferation and medial VSM cell disappearance in rat aortic allografts have been demonstrated to start at the same time (Ref. 32). According to the TA paradigm, the medial VSM cells migrate to the intima, resulting in an acellular media and the formation of a neointima. However, there is a striking loss of medial VSM cells by apoptotic cell death even before the development of a neointima (Ref. 79). It seems surprising that VSM cells proliferate at one side (neointima) of the vessel wall and die by apoptosis at another, nearby, location.

8

# **Recipient** origin

Besides a donor origin of neointimal ECs, repopulation of graft vessels by host-derived ECs has also been reported (Refs 80, 81), suggesting heterogeneity of the underlying processes.

With regard to VSM cells, alternative data suggest a host origin of  $\alpha$ -actin-positive VSM cells, which might be derived from the host bloodstream. After implantation in the aorta of young pigs, Dacron-hubs (Dacron discs placed in the bloodstream without having direct physical contact with the vascular wall) were covered with ECs and VSM-like cells that originated from cells in the blood (Ref. 82). Moreover, implantation of biodegradable compliant vascular grafts in rats resulted in development of new vascular wall structures, including a neomedia (with  $\alpha$ -actinpositive VSM cells) and a neointima (with ECs) (Ref. 83). In the Dacron-hubs as well as the synthetic biodegradable vascular grafts, the ECs and VSM cells should by definition be host derived, indicating that ECs and VSM cells can originate from the host. On the basis of these observations, it has been hypothesised that the development of TA in allogeneic grafts is in principle the same as in artificial vascular grafts, implying that neointimal ECs and VSM cells are host derived (Ref. 84).

To test this hypothesis, the origin of neointimal ECs and VSM cells was determined in two transplant models in rats: aortic and cardiac transplantation. Using MHC class I haplotypespecific immunohistochemistry it was shown that graft endothelium in non-immunosuppressed aortic allografts is indeed replaced by hostderived ECs (Ref. 85). In cardiac allograft recipients, in which alloreactivity was decreased although not completely abolished by intrathymic immune modulation (Ref. 86), graft endothelium was preserved (Ref. 85). These results indicate that whether or not graft endothelium is replaced by host-derived ECs depends on the severity of graft vascular damage. In line with this, a correlation between the level of EC replacement and the severity of vascular rejection has been recently shown in human kidney allografts (Ref. 87). It is therefore unlikely that EC replacement will improve long-term graft survival as suggested by Woodruff and by Medawar (Refs 74, 75). Since TA development was observed in both aortic allografts (EC replacement) as well as cardiac allografts (no EC replacement), 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.

In the aortic transplant model in rats, flow cytometry using allo-antisera suggested that the neointimal VSM cells were of host origin and not of donor origin (Ref. 32); however, neointimal VSM cells and neointima-infiltrating host-derived inflammatory cells could not be distinguished because of the procedure used. In a more recent study, the origin of neointimal VSM cells was therefore assessed using host-specific single-cell polymerase chain reaction (PCR) analysis on microdissected single VSM cell nuclei, and both in aortic and in cardiac allografts the VSM cells were of host origin (Refs 85, 88). Similar observations have been described in a femoral artery transplant model in rats (Ref. 89) and an aortic transplant model in mice (Refs 90, 91). It has also recently been shown in human kidney allografts that mesenchymal cells of host origin can be detected in the interstitial compartments of renal allografts with TA (Ref. 92). This suggests that circulating mesenchymal precursor cells (giving rise to fibroblasts, myofibroblasts and VSM cells) have the potential to migrate to areas of inflammation and contribute to the fibrotic process, eventually resulting in graft deterioration.

Host neointimal ECs and VSM cells might be derived from ingrowth from the recipient side of anastomosis or recruitment from a pool of circulating, possibly bone-marrow (BM)-derived, progenitor cells. Using BM-chimaeric rats, allowing discrimination between BM- and non-BM-derived cells, it has recently been shown that the host-derived ECs in advanced neointimal lesions in aortic allografts are primarily non-BM derived, indicating that a non-marrow source provides these cells (Ref. 93). The host-derived VSM cells were also predominantly, if not all, derived from a non-BM source. Although under certain circumstances BM-derived VSM cells and ECs can contribute to neointima formation and re-endothelialisation, respectively (Ref. 94), recent observations in a rtic transplant models in mice demonstrate that the percentage of BM-derived VSM cells is rather low (Refs 90, 91).

The precise anatomical origins of the hostderived non-marrow neointimal EC and VSM cells are as yet unknown. Migration of ECs from the recipient side of anastomosis cannot be excluded, but a pool of circulating ECs (Ref. 95) might be the main source. It has been suggested 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 has been observed (Ref. 96). Circulating VSM progenitor cells have not been identified so far. However, a population of non-BM-derived fibroblast-like cells in the peripheral blood (so called fibrocytes) that are specifically recruited from the blood to wounded areas has been described (Ref. 97). The differentiation and recruitment of fibrocytes is primarily mediated by TGF- $\beta$  and chemokines, and fibrocytes promote angiogenesis by the production of proangiogenic factors (Ref. 98). Based on these characteristics, fibrocytes might be the candidate cells responsible for TA development; however, this needs further study.

# Sequence of events leading to TA

The possible sequence of events before, during and after solid-organ transplantation that lead to the development of TA and subsequent CTD are summarised in Figure 2. A central element in the development of TA is injury of the graft vascular tree. Damage of the graft vasculature might occur before transplantation. For example, donor brain death and ischaemia after graft retrieval are the main causes of EC activation, thereby creating a pro-inflammatory milieu in the graft during the pre-transplant period. During the transplantation procedure, graft endothelium is further activated and might lose function mainly as a result of reperfusion injury to the graft. Most damage is, however, 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 (e.g. CMV infection) influence this process. This alloreactive response is responsible for damage to the graft ECs, and might result in replacement with host ECs. Moreover, as a result of the alloreactive response medial VSM cells die and disappear. In response to this, a process of uncontrolled neointimal proliferation of VSM cells is initiated, eventually resulting in the development of TA and CTD.

Figure 2. Pathogenesis of transplant arteriosclerosis and possible targets of therapeutic intervention. The scheme shows the proposed sequential events before and after solid-organ transplantation that lead to the development of transplant arteriosclerosis (TA) and chronic transplant dysfunction (CTD). Possible targets of therapeutic intervention to prevent transplant arteriosclerosis are indicated (a-e), and are discussed in more detail in the section entitled 'Possible targets for TA prevention and treatment'. Before transplantation and during the transplant procedure, graft endothelial cells (ECs) become activated and damaged as a result of alloantigen-independent factors such as donor brain death, ischaemia and reperfusion of the graft. Most of the damage, however, is inflicted after transplantation and the most important contributor in this process is the alloreactive response, which might be enhanced by cytomegalovirus (CMV) infections. Abolishing the alloreactive response by either use of improved immunosuppressive agents or the induction of transplant tolerance (a) are feasible strategies to reduce vascular damage in the graft, thereby preventing or slowing down the process of TA development. Additional treatment of acute CMV infections (b) might further reduce vascular damage. Damage of the graft EC lining might result in replacement of graft ECs with host-derived ECs, which most probably originate from a pool of non-bone-marrow (non-BM)-derived circulating endothelial cells (CECs). Whether or not graft endothelium will be replaced depends on the severity of vascular rejection (i.e. EC damage). EC replacement with host-derived ECs is an indicator for severe vascular damage but is not a prerequisite for the subsequent development of TA per se. In addition to EC damage, the medial vascular smooth muscle (VSM) cells are also damaged as a result of the alloreactive inflammatory response. Inflammatory cytokines and growth factors induce medial VSM cell apoptosis, resulting in disappearance of the medial VSM cells. Recruitment of host-derived VSM cells (primarily non-BM origin) occurs in an attempt to restore vascular wall function. However, neointimal VSM cells proliferate in an uncontrolled fashion, resulting in the development of TA and eventually CTD. Since medial VSM cell apoptosis appears to be an important event in initiating the recruitment of neointimal VSM cells, prevention of medial VSM cell apoptosis might be a possible target for therapeutic intervention (c). Uncontrolled proliferation of neointimal VSM cells is the main cause of luminal occlusion. Inhibition of the proliferative capacity of VSM cells by intervention strategies might result in decreasing the rate of TA development (d). The recruitment and proliferative capacity of neointimal VSM cells seems to be controlled by genetic factors, suggesting the existence of genetic predisposition for the development of TA and CTD. Identification of the gene products involved 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 (e) (see next page for figure) (fig002jhg).

10

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Figure 2. Pathogenesis of transplant arteriosclerosis and possible targets of therapeutic intervention *(see previous page for legend)* (fig002jhg).

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# TA: a normal vascular repair process beyond control?

Based on our own and other published data, we propose that the development of TA is a host repair process initiated to restore vascular wall function of vessels in which the medial architecture has been destroyed by an alloreactive inflammatory response (Ref. 84). The possible sequence of events in this repair process leading eventually to TA is depicted in Figure 3. Although the scheme presented is primarily based on observations made in experimental transplant models, this sequence of events might also apply to solid-organ transplants in clinical transplantation.

Graft ECs might be initially activated by the graft retrieval procedure, the transplantation procedure, and then the developing rejection response (characterised by perivasculitis and endothelialitis). The ongoing alloreactive response leads to further destruction of the EC lining of the graft, resulting in EC denudation. Concomittantly, medial VSM cells disappear as a result of apoptosis, leading to a complete absence of medial VSM cells and only the elastin scaffold of the media remaining. Subsequently, rebuilding of the damaged vessel wall occurs, consisting of reendothelialisation and VSM cell replacement. The initial appearance of VSM cells is followed by intimal hyperplasia caused by uncontrolled proliferation of VSM cells, eventually resulting in luminal occlusion.

The question remains why a healing process, which normally is self-limiting once function is fully restored, in TA does not stop and eventually leads 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 such as PDGF and bFGF are produced continuously. Alternatively, physiological shortcomings of the neointima to restore vascular wall function might be the driving force behind the ongoing intimal thickening observed in TA. This explanation is supported by observations made in biodegradable vascular grafts. Experiments in rats have shown that these grafts should be biocompatible, microporous, compliant and biodegradable (Ref. 99). 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 might essentially be the same as the process observed in the biodegradable compliant vascular grafts: as a result 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. A major difference between TA and biodegradable vascular grafts is that in the latter new elastic laminae developed within the neomedia with circularly oriented VSM cells (Ref. 83); in TA, however, neointimal VSM cells are randomly oriented and newly formed elastin is hardly present. A well-organised neomedia in biodegradable vascular grafts might restore graft function; the random organisation of neointimal VSM cells without elastic laminae in TA might not.

# Possible targets for TA prevention and treatment

Based on the data described in this review, several new targets of possible therapeutic intervention can be identified, which might be helpful in developing new strategies to prevent or treat TA in the future. In our opinion, strategies directed towards prevention of TA, rather than treatment of established TA, are most effective.

# Induction of transplantation tolerance

Damage induced by the alloreactive response appears to be the most important factor for TA development. Thus, deletion of host alloreactivity against the graft is an obvious solution to prevent TA after organ transplantation (Fig. 2a). New immunosuppressive drugs that abolish alloreactivity but do not have the toxic side effects of today's immunosuppressive agents (Box 1) might be effective in preventing TA.

It has been suggested that induction of immunological tolerance to the graft is the key to preventing CTD (Ref. 100). The creation of transplantation tolerance would obviate the need for long-term immunosuppression and theoretically could prevent CTD. Blockade of T-cell costimulation pathways not only results in suppression of the immune response, but has also been shown to be effective in inducing antigenspecific transplantation tolerance in rodents (Ref. 100). Optimal T-cell activation requires both antigen-specific signals (signal 1; interaction of Tcell receptor and MHC–peptide complex) and non-antigen-specific, costimulatory signals (signal 2) (Ref. 101). The best-understood costimulatory

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Figure 3. Revised view of the development of transplant arteriosclerosis after solid-organ transplantation *(see next page for legend)* (fig003jhg).

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Figure 3. Revised view of the development of transplant arteriosclerosis after solid-organ transplantation. The development of transplant arteriosclerosis (TA) and subsequent chronic transplant dysfunction (CTD) can be divided in five different phases. (a) Phase 1. Soon after transplantation (days to weeks), the vascular wall is infiltrated with inflammatory cells as a result of host alloreactivity directed against the graft. This inflammatory response is characterised by perivasculitis (adventitial inflammation) and attack of graft endothelium by recipient inflammatory cells (endothelialitis). (b) Phase 2. The alloreactive response leads to destruction of the endothelial cell (EC) lining of the graft, resulting in EC denudation of the luminal surface of the vascular wall. Concomittantly with the disappearance of the EC lining, medial vascular smooth muscle (VSM) cells start to disappear as a result of apoptosis due to the ongoing inflammation in the adventitia. Complete absence of the medial VSM cells is observed, with only the elastin scaffold remaining. (c) Phase 3. Rebuilding of the damaged vessel wall, which has lost appropriate function, starts: a new EC layer of recipient origin (re-endothelialisation) is built both by ingrowth of recipient ECs over the denuded intima starting at the side of anastomosis as well as by recruitment of circulating ECs, which may originate from a non-bone marrow source (such as the host vascular wall). (d) Phase 4. VSM cell replacement starts either through ingrowth of recipient VSM cells into the graft intimal space starting at the side of the anastomosis, or from circulating, non-bone marrow derived, recipient VSM (progenitor) cells. (e) Phase 5. Initial appearance of VSM cells is followed by intimal hyperplasia caused by uncontrolled proliferation of VSM cells, resulting in luminal occlusion (TA) and eventually CTD (fig003jhg).

pathway is provided through the T-cell surface molecule CD28 and its ligands CD80 (B7-1) and CD86 (B7-2) on antigen-presenting cells (APCs) (Ref. 102). Blockade of this costimulatory CD28-B7 pathway early after transplantation by using CTLA4-Ig fusion protein reduces development of TA and other, graft-specific, histopathological findings of CTD in cardiac and kidney allografts in rodents (Refs 103, 104). Even blocking CD28-B7-mediated T-cell costimulatory activation late after transplantation, after initial graft injury, has been shown to prevent progression of CTDrelated histopathology (Ref. 105). When CTLA4– Ig treatment was suboptimal, and antidonor reactivity (partly) remained, the development of TA could not be prevented (Ref. 106).

Another T-cell costimulatory pathway is mediated through CD40, expressed on a variety of cell types including APCs, B cells and ECs, and its ligand CD154, which is expressed on activated T cells (Ref. 100). Although blockade of the CD40–CD154 pathway using monoclonal antibodies prolonged graft survival, its effect on the development of CTD-related histopathology remains controversial (Refs 107, 108). Moreover, cardiac allografting in CD154<sup>-/-</sup> knockout mice did not reduce the development of TA (Ref. 109). Since TA is primarily initiated by alloreactive T cells, it is predictable that suppressing T-cell alloreactivity inhibits the development of TA several weeks to months after transplantation (which are the end-points in most animal studies). However, in clinical practice, transplant recipients need life-long treatment with immunosuppressive drugs, and so far it is not known whether the new immunosuppressive drugs and costimulation blockers also prevent TA development in the longterm. Therefore, TA deceleration should not be confused with TA elimination (Ref. 6).

Donor-specific immunological non-reactivity can also be established by the induction of mixed chimaerism after allogeneic BM transplantation. Donor-specific BM transplantation in mice has been shown to induce permanent graft acceptance of allogeneic skin and cardiac grafts. Although mixed chimaerism was achieved, development of TA in cardiac allografts was not prevented, suggesting that donor reactivity was not completely abolished by this treatment (Ref. 110).

Decreasing immunological reactivity by intrathymic immune modulation is effective in preventing acute rejection of cardiac allografts in rodents (Ref. 111). However, although preventing acute rejection and prolonging graft survival, intrathymic immune modulation did not prevent TA development in allogeneic cardiac allografts (Ref. 86). Intrathymic immune modulation did not completely abolish alloreactivity but rather induced immune deviation.

Most tolerance-inducing protocols 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 protocols that do not eliminate alloreactivity completely, but rather suppress the immune response against the graft, will fail to prevent the eventual development of TA. According to this view, only those interventions in which anti-donor reactivity is completely blocked or deleted [i.e. 'true tolerance' – for example, the creation of complete donor-BM chimaeras (Refs 112, 113)],

will prevent the development of TA. Indeed, in a recent case report it has been shown that induction of true (complete) chimaerism by donor-specific BM transplantation might lead to indefinite renal allograft survival without CTD in the absence of chronic immunosuppression (Ref. 114). However, BM 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.

### Antiviral chemotherapy

Active CMV infection in transplant recipients might result in CMV disease ranging from general discomfort to a severe, often life-threatening, wasting disease (Ref. 115). Moreover, active CMV infection can have indirect effects such as enhancement of the development of TA in susceptible patients. Patients at high risk for active CMV infection generally are seronegative recipients receiving an allograft from a seropositive donor. Moreover, the amount of immunosuppression necessary to prevent acute rejection is an important risk factor for active CMV infection. Seronegative recipients receiving a lung or intestine allograft from a seropositive donor require more immunosuppression than do liver, heart and kidney allograft recipients and are therefore at higher risk for active CMV infection (Ref. 115). To prevent direct (CMV disease) and indirect (TA) effects of CMV, active CMV infection should be prevented (Fig. 2b). This can be achieved by pre-emptive therapy with ganciclovir [i.e. therapy started at the time of detection (by antigenaemia or quantitative PCR) of early CMV replication] in an attempt to prevent progression of asymptomatic infection into CMV disease. Another strategy is prophylactic use of ganciclovir (i.e. administration of ganciclovir from the earliest possible moment). It has been shown that prophylaxis can have an inhibitory effect on the development of TA in seronegative cardiac allograft recipients, even without reduction of CMV disease (Ref. 116). So, the mechanism by which ganciclovir prophylaxis might reduce TA is still unclear and might be independent of the inhibitory effect on viral replication or CMV disease. A direct effect of ganciclovir on neointimal VSM cell replication, as shown on cultured VSM cells in vitro (Ref. 60), can therefore not be excluded.

Given that prophylaxis has some serious disadvantages compared with pre-emptive therapy [therapy is costly, exposes all patients to this potentially toxic drug and leads to development of late CMV infection and disease in high-risk patients (Ref. 117)], pre-emptive ganciclovir therapy should be recommended for prevention of active CMV infection and disease, except for high-risk patients. Although prevention of active CMV infection by either pre-emptive therapy or anti-CMV prophylaxis by itself will not prevent development of TA, it might be effective in reducing the rate and severity of TA development.

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 (Fig. 2c). Since medial VSM cells disappear as a result of apoptosis (Ref. 79), therapies directed at prevention of medial VSM cell apoptosis might be effective in the prevention of TA. This should be achieved indirectly by improved immunosuppression or tolerance induction, but, as discussed, these strategies have had limited success. Direct prevention of medial VSM cell apoptosis should also be a feasible strategy. To our knowledge, however, this strategy has not been studied in experimental or clinical studies so far.

Once the medial VSM cells have disappeared and host-derived VSM cells have been recruited to form the neointima, a possible intervention is prevention of VSM cell proliferation beyond the stage of functional repair (Fig. 2d). Several, mainly experimental, approaches have been followed to target the proliferative capacity of VSM cells. Sirolimus (rapamycin) exerts antiproliferative actions on cultured VSM cells in vitro, besides its known immunosuppressive effects. Moreover, this drug disrupts signal transduction pathways induced by a variety of cytokines and growth factors (Ref. 118). Because of its immunosuppressive effects as well as its antiproliferative capacities, Sirolimus might be useful as a treatment or prophylaxis for TA.

In addition, the HMG-CoA reductase inhibitor pravastatin shows, besides its cholesterollowering and immunosuppressive actions, a direct inhibitory effect on VSM cell proliferation (Ref. 119), and antihypertensive drugs (e.g. the calcium-channel blocker diltiazem and the ACE inhibitor captopril) and the somatostatin analogue angiopeptin seem to inhibit VSM cell proliferation (Refs 119, 120, 121). Although experimental data on the antiproliferative effects of angiopeptin have been described, the beneficial effect of angiopeptin on TA has still to be proven.

One promising drug effective in reducing VSM cell migration and proliferation is tranilast, a drug originally discovered as an anti-allergic agent inhibiting cytokine production from mast cells and macrophages. Clinical data show that treatment with tranilast prevents restenosis after balloon-angioplasty and coronary stenting (Ref. 122). Recently, tranilast was also shown to inhibit the development of TA in a mouse heart transplant model (Refs 123, 124). Both experimental studies showed a decreased number of neointimal proliferating cells following tranilast treatment. Moreover, tranilast seemed to be selective for cell proliferation of graft neointimal cells, whereas physiologically normal cell proliferation (e.g. spermatogenesis, intestinal cell proliferation) was not inhibited by tranilast (Ref. 124). Since neointimal VSM cells have now been shown to be predominantly host-derived (Refs 85, 90), it is feasible that tranilast affects homing, differentiation and proliferation of putative smooth muscle progenitor cells derived from the host (Ref. 123).

Induction of intimal VSM cell apoptosis is another strategy to prevent TA development. In a mouse heart transplant model, intragraft blocking of Bcl-x expression (an anti-apoptotic mediator) by antisense Bcl-x oligodeoxynucleotide was effective in preventing intimal hyperplasia through enhancing apoptosis of neointimal VSM cells (Ref. 125). Interestingly, transfection with antisense Bcl-x throughout the entire allograft resulted in apoptosis only in the Bcl-x-positive cells. Only proliferating neointimal VSM cells express Bcl-x; medial VSM cells as well as ECs and myocytes do not. General transfection thus results in selective induction of apoptosis of neointimal VSM cells and suppression of TA in the coronary arteries.

# Identification of high-risk patients

Since genetic differences between organ transplant recipients influence 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 might provide new tools for therapeutic intervention. Immunosuppressive regimens could be adjusted for such patients at high risk for developing TA (Fig. 2e).

### **Concluding remarks**

Our understanding of the risk factors in the pathogenesis of TA, and the precise mechanisms leading to vascular narrowing, is continually improving. Prevention of severe vascular wall damage seems to be the key element in preventing TA and subsequent CTD. Therefore, the development of new, more-potent and lesstoxic immunosuppressive agents is desirable. Moreover, induction of true transplant tolerance should be a feasible strategy to prevent TA. If vascular wall damage occurs, inhibition of uncontrolled proliferation of neointimal VSM cells and/or induction of apoptosis in these cells might be possible targets for therapeutic intervention.

Thus, although development of TA and CTD is still a major problem after clinical organ transplantation, recent clinical and experimental data are revealing possible new targets for intervention. This knowledge provides a basis for future studies in the struggle against TA after solid-organ transplantation.

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Chronic transplant dysfunction and transplant arteriosclerosis

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# Further reading, resources and contacts

The United Network for Organ Sharing (UNOS) maintains an organ transplant waiting list and organises donor-graft allocation within the USA:

http://www.unos.org

The International Society for Heart and Lung Transplantation (ISHLT) promotes research and treatment of end-stage heart and lung diseases:

http://www.ishlt.org

The Eurotransplant International Foundation is responsible for organ donation procedures in Austria, Belgium, Germany, Luxembourg, the Netherlands and Slovenia:

http://www.eurotransplant.org

The Transplant Pathology Internet Services (TPIS) (Division of Transplantation Pathology, University of Pittsburgh, PA, USA) and the organ transplantation section of eMedicine provide general information about solid-organ transplantation and photomicrographs of graft histology:

http://tpis.upmc.edu/ http://www.emedicine.com/med/TRANSPLANTATION.htm

### http://www.expertreviews.org/

# Features associated with this article

### **Figures**

Figure 1. The histopathology of transplant arteriosclerosis (fig001jhg).

Figure 2. Pathogenesis of transplant arteriosclerosis and possible targets of therapeutic intervention (fig002jhg).

Figure 3. Revised view of the development of transplant arteriosclerosis after solid-organ transplantation (fig003jhg).

# Table

Table 1. Incidence, clinical presentation and histopathology of chronic transplant dysfunction (CTD) after solid-organ transplantation (tab001jhg).

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