Recipient Origin of Neointimal Vascular Smooth Muscle Cells in Cardiac Allografts with Transplant Arteriosclerosis

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Abstract

**Background.** Coronary artery disease is today’s most important post-heart transplantation problem after the first perioperative year. Histologically, coronary artery disease is characterized by transplant arteriosclerosis. The current view on this vasculopathy is that vascular smooth muscle (VSM) cells from the media of affected arteries proliferate and migrate into the subendothelial space (intima) in response to signals from inflammatory cells and damaged graft endothelium. According to this model, the intimal VSM cells in transplant arteriosclerotic lesions should originate from donor tissue. Using recipient-specific polymerase chain reaction (PCR) analysis of microdissected, single, neointimal VSM nuclei, we recently showed that after allogeneic aorta transplantation the neointimal VSM cells are of recipient and not of donor origin. In this study, we analyzed whether VSM-cell replacement with recipient-derived cells also takes place after allogeneic heart transplantation.

**Methods.** Cardiac allografts, when transplanted from female donors to male immune-modulated recipient rats, eventually developed transplant arteriosclerosis. We microdissected α-actin positive neointimal VSM cells from tissue sections and determined the origin (donor or recipient) using recipient (male), single-cell, PCR analysis.

**Results.** In total, we analyzed 35 VSM-cell nuclei from 3 allografts, and PCR analysis revealed that 30/35 (86%) of the samples displayed the male-specific 128 base pair DNA fragment. These results indicate that after allogeneic cardiac transplantation, at least 86% of VSM cells in transplant arteriosclerotic lesions are of recipient origin.

**Conclusions.** In contrast to current thought, the neointimal VSM cells in cardiac allografts that show transplant arteriosclerosis are of recipient and not of donor origin.
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Introduction

Development of chronic transplant dysfunction (CTD) is the primary cause of allograft loss after the first perioperative year and is today’s most important problem in clinical organ transplantation. Current immunosuppressive protocols cannot prevent CTD. Depending on the type of organ grafted (liver, kidney, heart or lung), the incidence of CTD varies from 5% in liver allografts to >50% in lung transplants. After clinical heart transplantation, coronary artery disease (CAD) is the main cause of CTD. Five years after heart transplantation, CAD is present in about 40% of transplant recipients.

Coronary artery disease is characterized by a process of vascular remodeling referred to as transplant arteriosclerosis, and consists of ongoing perivascular inflammation and progressive intimal thickening (intimal hyperplasia). This vasculopathy is generally accepted as the main cause for progressive deterioration in graft function after heart transplantation. Mechanisms involved in the development of CAD development, however, remain obscure. Risk factors appear to include alloantigen dependent factors (e.g., major histocompatibility complex [MHC] disparity and the number of acute rejection episodes) as well as alloantigen independent factors (e.g., donor age, donor hypertension, cold ischemia/reperfusion injury, and cytomegalovirus infection).

The current view on transplant arteriosclerosis is that donor-derived medial vascular smooth muscle (VSM) cells of affected arteries start to proliferate and migrate into the sub-endothelial space (intima) in response to signals from inflammatory cells (perivasculitis) and damaged graft endothelium. According to this model, intimal smooth muscle cells in transplant arteriosclerotic lesions should originate from donor tissue. Presently, however, few experimental or clinical studies have addressed the question of whether the VSM cells in intimal lesions are indeed graft-derived (donor), and results conflict. Using recipient-specific polymerase chain reaction (PCR) analysis of microdissected, single, neointimal VSM cell nuclei, we recently showed that, after allogeneic aorta transplantation in rats, neointimal VSM cells are (for the vast majority) of recipient and not of donor origin. We hypothesized that development of transplant arteriosclerosis is an attempt to restore vascular wall function after immunologic injury and essentially is part of a normal healing response. In contrast to the normal healing response, which stops after healing, the remodeling process in transplant arteriosclerosis does not seem to stop, and eventually leads to total occlusion of the vessels involved.

Because transplantation of aortic allografts provides a simplified model to study pathogenesis of transplant arteriosclerosis, we wondered whether VSM-cell replacement is a general phenomenon after transplantation and not unique to vascular allografts. Therefore, we analyzed the origin (donor vs. recipient) of VSM cells in cardiac allografts that were transplanted in an MHC-incompatible rat strain combination after intrathymic (IT) immune modulation of the recipient rats. Intrathymic immune modulation prevents acute rejection and significantly prolongs graft survival. However, this strategy cannot prevent transplant arteriosclerosis development.
single cell, PCR analysis of microdissected neointimal VSM-cell nuclei, we show that also after cardiac allografting, the neointimal VSM cells throughout the graft are of recipient origin.

**Materials and Methods**

*Animals*
We obtained specific pathogen-free female PVG (RT-1\(^c\)) and male AO (RT-1\(^u\)) rats from the Central Animal Facility of the Faculty of Medical Sciences of the University of Groningen. The PVG and AO rats were age-matched (7 to 10 weeks of age) and used as donors and recipients, respectively. We maintained animals under clean, conventional conditions and fed them standard rat chow and acidified water *ad libitum*. All animals received humane care in compliance with the *Principles of Laboratory Animal Care* (NIH Pub. No. 86-23, revised 1985) and the Dutch Law on Experimental Animal Care.

*Cardiac Transplantation and IT Immune Modulation*
To prevent acute rejection after cardiac allografting in the fully MHC-incompatible PVG-to-AO rat-strain combination, we used a protocol developed in our laboratory and described elsewhere\(^{19}\) (Group 1, n=39). Cardiac allografts thus transplanted eventually develop transplant arteriosclerosis indicative of CTD. Control groups consisted of rats that received cardiac allografts and subsequent treatment with rabbit anti-rat lymphocyte serum (ALS) and cyclosporine (CsA) (Group 2, n=8) or that received no further treatment at all (acute rejection, Group 3, n=9). We monitored grafts until rejection (cessation of palpable ventricular contraction). We also transplanted AO grafts to serve as isograft controls (Group 4, n=3). All grafts promptly started beating after revascularisation, and we regularly assessed graft function by palpation.

*Evaluation of Transplant Arteriosclerosis*
To analyze presence of transplant arteriosclerosis after IT modulation (Group 1), we removed functioning (beating) grafts at various points after transplantation (ranging from 100 to 500 days after transplantation). We did not include grafts from Group 2 (CsA and ALS treatment only) in this histologic analysis. To study vasculopathy in acutely rejecting allografts, we transplanted 3 additional grafts, gave them no further treatment, and removed grafts 4 days after transplantation (at this time the grafts were still beating) (Group 3). We removed isografts (Group 4) 250 days after transplantation. We embedded grafts in paraffin and processed them for histologic analysis. Development of transplant arteriosclerosis was evaluated on tissue sections stained with orcein (Gurr, BDH Chemicals Ltd.; UK) for elastin. To confirm presence of intimal VSM cells in neointimal lesions, we performed a double staining for elastin and VSM-cell \(\alpha\)-actin\(^{20,21}\).

*Microdissection of Single Cells from Tissue Sections*
To study the origin (donor vs. recipient) of neointimal VSM cells in transplant arteriosclerosis, we first microdissected cell clusters (20 to 50 cells) from neointimal lesions from 5 grafts (showing severe transplant arteriosclerosis) using a micro-manipulator (Leica, Germany). However, transplant arteriosclerotic lesions not
only contain α-actin-positive VSM cells, but also (recipient derived) infiltrating leukocytes (T cells and macrophages). Therefore, we also microdissected single nuclei from α-actin-positive VSM cells to prevent potential contamination with neointima infiltrating cells. We used α-actin staining to ensure positive identification of VSM cells. From the 5 grafts used for cell-cluster microdissection, we selected 3 grafts for microdissection of single nuclei. For each graft studied, nuclei were isolated from at least 2 representative cross sections (4 nuclei/section). Nuclei were randomly selected from among α-actin-positive cells that had well-visible nuclei surrounded by α-actin-positively stained cytoplasm. Isolated nuclei were directly transferred into PCR tubes containing MilliQ water and stored at −20°C until PCR analysis.

Single-Cell PCR Analysis
Because we performed all transplantations in female (donor) to male (recipient) combinations, we could use sex markers as indicators for cellular origin. Therefore, we developed a highly sensitive and specific, nested PCR procedure that enabled identification at the single-cell level of a Y-chromosome-associated DNA sequence and thereby identification of the (male) origin of cells. We designed 2 sets of primers (for the first- and second-round PCR) to detect specifically male-derived cells using the Y-chromosome-specific sequence as described by Essers et al.22. These sequence data are available from EMBL/Genbank Data Libraries under accession number X80155 (clone 9.1ES8). The sequences of the first-round primers were 5’-CAGGCTGGAATGTTCCGATTACGGACTTG-3’ (forward) and 5’-CCCATGTTTGTCACCATAG-3’ (reverse), and the sequences of the second-round primers were 5’-GCTGGTGATTTCAGAGTGA-3’ (forward) and 5’-GAAATGTTCCGACCGCTG-3’ (reverse). The first and second rounds of PCR amplification resulted in 549 base pair (bp) and 128 bp DNA fragments, respectively. Before amplification, single nuclei were treated with proteinase K (Boehringer Mannheim, Germany) for 1 hour at 50°C, and thereafter proteinase K was heat inactivated for 10 minutes at 95°C. First and second rounds of PCR reactions were performed in a 50 µl mixture containing 1 U Taq polymerase (Ther- mus aquaticus YT1), 200 µmol/liter dNTP, 30 µmol/liter of each of the two primers (forward and reverse), 0.05% W-1, and 1.5 mmol/liter MgCl₂ in PCR buffer (Life Technologies B.V.; The Netherlands). Temperature profile for the first round of PCR follows: 3 minutes denaturation at 95°C; 35 cycles of 60 seconds at 95°C, 60 seconds at 60°C, and 80 seconds extension at 72°C; 7 minutes extension at 72°C. After the first round of PCR, 2 µl of the first-round reaction mixture was transferred into a new reaction tube containing second-round reaction mixture. Temperature profile for the second round of PCR follows: 3 minutes denaturation at 95°C; 30 cycles of 60 seconds at 95°C, 60 seconds at 60°C, and 80 seconds of extension at 72°C; 7 minutes extension at 72°C. Amplifications were carried out using an Amplitron® II Thermolyne thermocycler (Barnstead/Thermolyne, USA). After the second round of PCR, 5 µl of the reaction mixture was analyzed on a 2% agarose gel. Presence of the 128 bp fragment indicates male (recipient) origin of the nucleus analyzed.
Statistical Analysis
To analyze differences in graft survival rates for statistical significance, we performed a 2-tailed Mann-Whitney U Test using Graphpad Prism™ 2.0.1 for Windows. Differences were considered statistically significant when \( P < 0.05 \).

Results

Graft Survival After IT Immune Modulation
Intrathymic inoculation of donor splenocytes followed by short-term immunosuppressive treatment (Group 1) resulted in significantly prolonged graft survival \( (P < 0.0001) \) (Figure 1). Median survival time after IT inoculation was >208 days compared with 20 days after CsA and ALS treatment without IT inoculation (Group 2), and with 8 days in acute rejec-

Figure 1. Graft survival after intrathymic immune modulation. Intrathymic inoculation of 2.5x10^7 donor splenocytes and short-term immunosuppressive treatment (Group 1, \( n=39 \)) results in significantly prolonged graft survival of MHC-incompatible cardiac allografts compared with grafts transplanted in recipients treated with ALS and CsA only (Group 2, \( n=8 \)) and compared with acutely rejecting grafts (Group 3, \( n=9 \)) \( (P < 0.0001, \) Mann Whitney U test). Only 4 of 39 allografts were rejected, whereas the others showed ‘indefinite’ survival and remained beating until removal. Cardiac isografts (Group 4, \( n=3 \)) showed indefinite survival and remained beating until removal 250 days post-transplantation. Open circles represent cardiac grafts in respective groups that remained beating until removal, whereas the closed circles represent rejected grafts. ALS, rabbit anti-rat lymphocyte serum; AR, acute rejection; CsA, Cyclosporin A; IT inoc., intrathymic inoculation.
tion (Group 3). Treatment with CsA and ALS alone resulted in significantly prolonged graft survival compared with acute rejection ($P<0.0001$). Only 4 out of 39 cardiac allografts transplanted after IT inoculation were rejected (at time points comparable with animals treated with CsA and ALS only, Group 2). Grafts that were not rejected after IT inoculation survived ‘indefinitely’, and we removed them for histologic analysis at several time points (ranging from 100 to 500 days) after transplantation, still beating at that time. Cardiac isografts also survived indefinitely and were beating at removal (mean survival time >250 days).

**Development of Transplant Arteriosclerosis**

Intrathymic inoculation prevented rejection of cardiac allografts in the majority of the grafts transplanted (Figure 1). However, IT inoculation did not prevent the development of transplant arteriosclerosis, indicative of CAD. In beating grafts that were removed before 200 days after transplantation, up to 36% ± 25% of coronary arteries showed moderate to severe transplant arteriosclerosis (i.e., circumferential intimal thickening with > 20% luminal compromise). In grafts removed between 200 and 500 days after transplantation, 80% ± 18% of the coronary arteries showed severe transplant arteriosclerosis, in which the vascular lumen was completely occluded. We observed a positive correlation between the severity of transplant arteriosclerosis and the time of explantation. Immunohistochemical staining revealed large numbers of α-actin-positive VSM cells in neointimal lesions of coronary arteries, showing severe transplant arteriosclerosis.

In cardiac isografts, explanted 250 days after transplantation, coronary arteries were virtually free from severe transplant arteriosclerosis and generally appeared normal. Allografts without further treatment (Group 3) were rejected (cessation of palpable ventricular contraction) within 7 days after transplantation. Therefore, we transplanted another 3 allografts and gave no further treatment. These remained functional until removed 4 days after transplantation. We used these grafts for further histologic analysis. We observed no transplant arteriosclerosis and blood vessel pathology in rejecting allografts was characterized by mononuclear-cell infiltration of the intima (without presence of α-actin-positive cells), often coinciding with perivascular infiltration. Thus, IT inoculation of donor splenocytes followed by short-term immunosuppressive therapy prevents (acute) rejection but not development of transplant arteriosclerosis. Figure 2 shows representative micrographs of the morphologic appearance of coronary arteries in the various treatment groups.

**Single-cell PCR Analysis of VSM Cells**

Because we performed cardiac allografting not only between MHC-incompatible rat strains but also in a female (donor) to male (recipient) combination, we could use sex markers as indicators for cellular origin. Therefore, a highly sensitive and specific, nested PCR procedure was developed that enabled the identification, at the single-cell level, of a 128 bp Y-chromosome-associated DNA sequence and thereby the (male) origin of cells. Specificity and sensitivity of this PCR was confirmed by PCR analysis on serially diluted male and female splenocyte-derived
Figure 2. Photomicrographs of coronary artery morphology after intrathymic immune modulation and in the control groups. Staining for VSM-cell α-actin shows medial and neointimal α-actin-positive VSM cells. In normal (non-transplanted) cardiac tissue as well as cardiac isografts (250 days after transplantation), no blood vessel pathology was present (A and B). Acutely rejecting allografts showed vascular rejection with disruption of the vascular media, but without transplant arteriosclerosis (C). After intrathymic immune modulation (>200 days after transplantation), the vast majority of blood vessels showed severe transplant arteriosclerosis with (nearly) complete luminal occlusion. The neointimal lesions mainly consisted of α-actin-positive cells (D). IEL, internal elastic lamina; LU, lumen; M, media; NI, neointima (original magnification, x400).
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DNA as well as PCR analysis on single-cell sorted (using a FACS® cell sorter) male and female thymocytes. In both analyses, the PCR proved to be male specific and sufficiently sensitive to detect male-derived cells at the single-cell level (not shown). We first performed PCR analysis on cell clusters that we micro-dissected from neointimal lesions from 5 grafts that showed severe transplant arteriosclerosis (majority of the coronary arteries completely obliterated), explanted 436, 469, 469, 481 and 481 days after transplantation. Polymerase chain reaction analysis showed that recipient-derived cells were present in the cell clusters (not shown). To prevent potential contamination with neointima infiltrating recipient-derived leukocytes, we subsequently performed single-cell PCR analysis on VSM-cell nuclei microdissected from 3 selected grafts, which were ex-

![Microphotograph showing double staining for VSM cells (α-actin) and elastin (Lawson) of a coronary artery with severe transplant arteriosclerosis after microdissection of single, VSM cell nuclei using a micro-manipulator. White arrowheads indicate α-actin-positive VSM cells, whereas black arrowheads indicate remaining gaps after dissection of nuclei of 2 α-actin-positive VSM cells (original magnification x400).](image1.png)

![Eight samples, representing 8 single nuclei from α-actin-positive cells dissected from 2 cardiac allografts transplanted from female-donor to male-recipient rats, showing the Y-chromosome-specific (recipient) 128 bp DNA fragment. bp ladder, 100 base pair ladder; H2O, water control (i.e. no nucleus present). NI, neointima; IEL, internal elastic lamina.](image2.png)

**Figure 3.** Vascular smooth muscle (VSM) cells in neointimal lesions are of recipient origin. (A) Microphotograph shows double staining for VSM cells (α-actin) and elastin (Lawson) of a coronary artery with severe transplant arteriosclerosis after microdissection of single, VSM cell nuclei using a micro-manipulator. White arrowheads indicate α-actin-positive VSM cells, whereas black arrowheads indicate remaining gaps after dissection of nuclei of 2 α-actin-positive VSM cells (original magnification x400). (B) Eight samples, representing 8 single nuclei from α-actin-positive cells dissected from 2 cardiac allografts transplanted from female-donor to male-recipient rats, showing the Y-chromosome-specific (recipient) 128 bp DNA fragment. bp ladder, 100 base pair ladder; H2O, water control (i.e. no nucleus present). NI, neointima; IEL, internal elastic lamina.
planted 436, 469 and 481 days after transplantation. 
α-Actin staining ensured positive identification of smooth muscle cells. Figure 3A shows a representative micrograph of a cardiac allograft with severe transplant arteriosclerosis after microdissection of 2 VSM-cell nuclei (black arrowheads).
We tested the efficacy of the microdissection procedure and subsequent PCR analysis on male-derived tissue sections, which turned out to be about 90%. From each graft, we microdissected at least 8 nuclei. In total, 35 individual nuclei were isolated and analyzed. Polymerase chain reaction analysis revealed that 30 of 35 nuclei analyzed displayed the male-specific 128 bp DNA fragment, whereas 5 (DNA containing) samples were negative for the male-specific 128 bp DNA fragment. This high frequency of positive samples indicates that at least 86% of VSM cells in (severe) transplant arteriosclerotic lesions after allogeneic cardiac transplantation are of recipient origin. Because the efficacy of the microdissection procedure and subsequent PCR analysis was about 90%, the results indicate that virtually all neointimal α-actin positive cells are of recipient origin. We dissected nuclei from neointimal lesions from large (primary) coronary arteries as well as from small coronary arteries deep in the myocardial tissue, indicating that not only VSM cells near the site of anastomosis but that all VSM cells throughout the cardiac graft are of recipient origin. Figure 3B shows representative results of the Y-chromosome-specific, single-cell PCR analysis performed on microdissected VSM-cell nuclei from 2 different cardiac allografts.

Discussion

Coronary artery disease is the main cause of long-term transplant dysfunction and affects up to 40% of heart transplant recipients 5 years after transplantation6,7. This vasculopathy is characterized by perivascular inflammation, disappearance of medial VSM cells, and a generalized concentric intimal thickening (transplant arteriosclerosis), consisting of vascular smooth muscle (VSM) cells intermingled with some T cells and macrophages2. Current thought on the process of transplant arteriosclerosis holds that medial VSM cells of affected arteries in the graft proliferate and migrate into the sub-endothelial space (intima) and therefore are donor derived8-11. Presently, however, few studies have addressed the question of whether the VSM cells in intimal lesions are indeed graft-derived and results conflict12-15. Using specific immunohistochemistry and recipient-specific, single-cell PCR analysis, we recently showed that after allogeneic aorta transplantation in rats, the endothelial cells as well as neointimal VSM cells are of recipient and not of donor origin16. These observations are in line with results reported by others, who also used models in which allogeneic vascular grafts were transplanted to study the origin of neointimal cells13,14. Because transplantation of a vascular allograft provides a simplified model to study the pathogenesis of transplant arteriosclerosis, we wondered whether VSM-cell replacement with recipient derived cells is a general phenomenon after allogeneic transplantation or is strictly related to vascular remodeling after transplantation of (large) vascular allografts. Recipient origin of VSM cells after solid
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organ transplantation has not been reported so far. Using another rat model, we analyzed the origin (donor vs. recipient) of neointimal VSM cells in cardiac allografts showing severe transplant arteriosclerosis. In this model, IT immune modulation prevented acute rejection, eventually resulting, however, in the development of transplant arteriosclerosis. Using recipient-specific, single-cell PCR analysis of microdissected neointimal VSM-cell nuclei, we, for the first time, show that also after solid-organ transplantation (cardiac allograft) the neointimal VSM cells are of recipient origin. Cardiac allografts surviving >200 days displayed severe transplant arteriosclerosis and 86% of the microdissected neointimal VSM cells were of recipient origin. Because the efficacy of the microdissection procedure and subsequent PCR analysis was about 90%, the results indicate that neointimal VSM cells are essentially recipient-derived.

Thus, not only in vascular transplantation but also in solid-organ transplantation, the VSM cells in neointimal lesions are predominantly and most likely entirely of recipient origin, indicating VSM-cell replacement during the development of transplant arteriosclerosis in such grafts. Whether VSM-cell replacement by recipient cells is a general phenomenon after solid-organ transplantation needs further investigation. We propose that sufficient initial damage to the graft endothelium has to occur (as a result of alloreactivity but also as a result of ischemia/reperfusion injury) before replacement of graft endothelial and as well as VSM cells will take place. This might explain why Hruban et al and Bittmann et al studying human cardiac and renal allografts respectively, reported donor origin of neointimal VSM cells and endothelium in grafts that showed transplant arteriosclerosis. Perhaps in these grafts, endothelial and VSM-cell replacement did not occur because of a lack of severe initial vascular damage. In the absence of severe damage of donor endothelium, endothelial cells will not be replaced by recipient-derived endothelial cells. However, prevention of donor endothelial-cell replacement does not automatically prevent development of transplant arteriosclerosis. Hruban et al and Bittmann et al clearly observed arteriosclerotic blood vessels that contained donor-derived VSM cells. Taken together, these results suggest that in the absence of donor endothelial-cell replacement, transplant arteriosclerosis, containing donor-derived (possibly media) VSM cells, can still develop.

In allogeneic aorta transplantation, on the other hand, donor endothelium disappears within 15 days after transplantation and is replaced by recipient endothelial cells at day 18. Also medial (donor) VSM cells have disappeared within 25 days after transplantation. These observations suggest severe damage of donor endothelial cells as well as VSM cells, leading to replacement with recipient-derived endothelial cells and neointimal VSM cells. Also in our model of allogeneic cardiac transplantation, graft endothelium and VSM cells are probably damaged to such an extent that eventually recipient-derived cells replace them.

Because this study shows neointimal VSM cells are recipient-derived, the question arises of the anatomic origin of these cells. Using combinations of MHC and gender-different bone marrow chimeric rats, we are presently investigating whet-
her these cells originate from the recipient side of anastomosis or from recirculating (possibly bone marrow-derived) recipient VSM progenitor cells. Recently, recirculating, bone marrow-derived endothelial progenitor cells have been identified in human peripheral blood\textsuperscript{24,25}, and seem to contribute to neovascularisation of ischemic regions\textsuperscript{26}. Circulating VSM progenitor cells have not been identified so far. However, Bucala et al reported the existence of a non-bone marrow derived circulating-cell population with fibroblast-like properties that specifically enters sites of tissue injury\textsuperscript{27}. These results strengthen the possibility that after severe donor endothelial damage, the endothelium as well as intimal VSM cells will be replaced by recipient-derived recirculating progenitor cells.

In conclusion, this study shows that also after solid-organ transplantation, the VSM cells in the hyperplastic neointima are of recipient and not of donor origin. We propose that development of allograft atherosclerosis is an attempt to restore vascular wall function after immunologic injury (alloreactivity), and is essentially part of the normal healing process\textsuperscript{18}. Basically, as a result of the immunologic injury, graft endothelium as well as medial VSM cells disappear, leaving the elastic network as a scaffold that allows restoration of the vessel wall by recipient-derived cells. In contrast to remodeling during normal healing, however, the process of vessel wall rebuilding after allogeneic grafting is obviously not properly controlled, resulting in luminal occlusion and graft dysfunction.

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References


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