EXAMINATION OF ENDOTHELIAL INTEGRITY IN A RAT CARDIAC TRANSPLANT MODEL: IMPLICATIONS FOR THE PATHOGENESIS OF TRANSPLANT VASCULAR DISEASE

by

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ABSTRACT

**Background:** One of the major complications associated with solid organ transplantation is a progressive form of allo-atherosclerosis known as transplant vascular disease (TVD), an expression of chronic allograft rejection. Events occurring early post-transplantation, particularly immune recognition of allo-endothelium, initiate TVD. Previous work has suggested an important compromise of endothelial integrity as the allo-immune milieu evolves, although mechanisms by which integrity is altered remain to be resolved. Increased vascular permeability due to endothelial damage may allow entrance of inflammatory cells, lipoproteins, other proteins and plasma fluid into the sub-endothelial space, thereby contributing to the initiation of atherosclerosis in thoroughfare arteries. In this study, endothelial integrity in coronary arteries and proximal aorta was examined following cardiac transplantation in rats.

**Hypothesis:** Altered endothelial integrity, as reflected in structural changes, is present in the rat allograft cardiac transplant model.

**Methods:** Lewis-to-Lewis and Lewis-to-F-344 rat heterotopic cardiac transplants were examined at 1, 4, 21 and 42 days post-transplantation. The effects of cyclosporine treatment (5mg/kg/day) on maintaining endothelial integrity were studied and compared with control saline treated animals. At the light microscopy level, *en face* silver nitrate staining was performed to demonstrate endothelial cell borders and gaps. Scanning electron microscopy was used to extend silver nitrate findings and to further define the presence and nature of endothelial disruptions. Transmission electron microscopy was performed to
further characterize endothelial integrity, immune cell identity and interaction with endothelium, and disease progression.

**Results:** Syngrafts and cyclosporine treated allografts showed normal looking endothelium similar to that observed in arteries from native and control hearts. However, saline treated allografts displayed progressive endothelial destruction including large intercellular gaps, missing cells, and areas of bare extracellular matrix. Exfoliated surfaces were covered by platelets at various stages of adhesion, activation and spreading. Similarly, numerous leukocytes were observed as either adherent to the endothelial lining or transmigrating into the sub-endothelial space. Cessation of cyclosporine therapy was associated with development of similar abnormalities.

**Conclusion:** These findings suggest that early endothelial damage may promote vascular permeability and thereby initiate TVD, especially when immunosuppression is insufficient.
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ABBREVIATIONS

APCs ........................................ Antigen presenting cells
AZA ........................................ Azathioprine
CAV ........................................ Cardiac allograft vasculopathy
CsA .......................................... Cyclosporine A
E-selectin ................................. Endothelial Selectin
F344 ......................................... Fisher
FK506 ....................................... Tacrolimus
GM-CSF ................................. Granulocyte macrophage-colony stimulating factor
HLA .......................................... Human leukocyte antigen
ICAM-1 ................................. Intercellular adhesion molecule-1
IFN-γ ................................. Interferon-gamma
IL-1 ........................................ Interleukin-1
IL-2 ........................................ Interleukin-2
IL-3 ........................................ Interleukin-3
IL-4 ........................................ Interleukin-4
IL-5 ........................................ Interleukin-5
IL-6 ........................................ Interleukin-6
IL-7 ........................................ Interleukin-7
IL-2R ........................................ Interleukin-2 receptor
i.m. ........................................... Intramuscular
I / R ........................................ Ischemia / reperfusion
ISHLT ................................. International Society for Heart and Lung Transplantation
i.v. ........................................... Intravenous
IVC .......................................... Inferior vena cava
IVUS ....................................... Intravascular ultrasound
LDL ........................................ Low density lipoprotein
LEW ........................................ Lewis
MHC ........................................ Major histocompatibility complex
MMF ........................................ Mycophenolate mofetil
NK ............................................ Natural killer
NFAT ........................................ Nuclear factor of activated T-cells
NF-ATc ..................................... Nuclear factor of activated T-cells cytolic subunit
NF-ATn ..................................... Nuclear factor of activated T-cells nuclear subunit
PDGF ........................................... Platelet derived growth factor
PMN ........................................... Polymorphonuclear leukocytes
RER ........................................ Rough endoplasmic reticulum
SEM ........................................ Scanning electron microscopy
SMC ........................................ Smooth muscle cell
TEM ........................................ Transmission electron microscopy
TNF-α ....................................... Tumor necrosis factor-alpha
TVD ........................................... Transplant vascular disease
VCAM-1 ..................................... Vascular cell adhesion molecule-1
VLDL ......................................... Very low density lipoprotein
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1  INTRODUCTION

1.1  HISTORY OF TRANSPLANT VASCULAR DISEASE (TVD)

Organ transplantation, including cardiac transplantation, is now a common procedure, with over 4000 cardiac transplants reported around the world each year (1, 2). The first successful experimental cardiac transplant surgery was reported forty-five years ago, pioneered by Dr. Norman Shumway and colleagues at Stanford University with studies of orthotopic transplantation using canine hearts (3). However, the first successful human-to-human orthotopic heart transplant would not occur until 7 years later by Dr. Christiaan Barnard and colleagues in Cape Town, South Africa on December 3 1967, using the Stanford technique (4). Although the first transplant recipient, Louis Washkansky, died after 18 days from complications of pneumonia, the second transplant performed one month later had a more favourable outcome and the patient conducted an active life for 19.5 months. The relative “success” from the second patient suggested that heart transplantation could become a viable treatment option for patients with end-stage heart disease. Unfortunately at autopsy, the transplanted heart showed signs of severe and significantly widespread coronary artery disease “so clearly visible without sectioning the vessels” (5). Every coronary artery from the main trunks to the smallest extramuscular branch was affected with vessels “literally stuffed with lipid so their external diameters were greatly increased” (5). As such, this was one of the first examples of an accelerated form of graft atherosclerosis known as cardiac allograft vasculopathy (CAV) or transplant vascular disease (TVD). Future transplants followed, and Bieber and
colleagues observed intimal infiltrate in longer surviving grafts containing host lipophages, usually localized on the intimal side of the internal elastic lamina among 12 human heart allografts (6). Therefore, it was already apparent at this time that TVD would become a major impediment to long-term graft survival. Almost forty years later, TVD is still the leading cause of long-term graft failure.

1.2 CLINICAL SIGNIFICANCE OF TVD

1.2.1 Incidence of TVD

For patients with end-stage disease, solid organ transplantation has become a common life saving procedure. In the overall International Society for Heart and Lung Transplantation (ISHLT) Registry experience (1982-2004), the primary indication for adult heart transplantation has been equally consistent between coronary heart failure and noncoronary cardiomyopathy (~45-47% respectively), with valvular (3-4%), adult congenital (2%), retransplantation (2%), and miscellaneous causes (1-2%) making up the remainder (Figure 1) (1, 2). Although the survival rate one-year post-transplantation among cardiac transplant patients is relatively high at 80-85%, it is dramatically reduced to 60-70% and 40-50% by five years and ten years post-transplantation, respectively (Figure 2) (1, 2). With the exception of acute rejection episodes and infections predominating within the first year post-transplantation, long term graft survival is still limited with the incidence of transplant mediated atherosclerosis, or TVD (7). Subsequently, graft half-life is just 9.6 years, and 12.0 years post-first year transplantation (Figure 2) (1, 2).
Figure 1: Patient diagnosis leading to the need for cardiac transplantation. CAD, coronary artery disease; Misc, miscellaneous; ReTx, retransplantation. From the 2005 Registry of the International Society for Heart and Lung Transplantation.
Figure 2: Kaplan-Meier survival for cardiac transplants performed between January 1982 and June 2004. Conditional half-life indicates 50% survival for patients surviving beyond the first year post-transplantation. From the 2005 Registry of the International Society for Heart and Lung Transplantation.
Transplant vascular disease is a unique and accelerated form of coronary disease that affects both intramyocardial and epicardial coronary arteries and veins (8). Although the disease selectively involves the vascular bed of the allograft including the donor aortic segment, all other native vessels of the host remain unaffected. This is dramatically illustrated at the lines of suture between the graft and host. Although there is evidence of partial reinnervation of the cardiac allografts, most cardiac transplant recipients do not experience the typical anginal pain (pectoris) associated with myocardial ischemia or infarction. Thus, acute symptoms may go unnoticed and patients may experience silent myocardial ischemia with the first clinical manifestations often consisting of ventricular arrhythmias, congestive heart failure, or sudden death. Using intravascular ultrasound (IVUS), intimal thickening within cardiac allografts can be detected in up to 75% of patients at one year post-transplantation (9), suggesting that development of TVD occurs very early after transplantation (10). Endothelial dysfunction also plays a key role in TVD and in native atherosclerosis. Moreover, endothelial dysfunction often precedes intimal thickening and subsequently, early endothelial dysfunction may predict the development of TVD during the initial year post-transplantation (11).

Histologically, TVD is a diffuse process starting with the small distal vessels and ultimately involving the intramyocardial and epicardial arteries of the allograft (12) with concentric intimal hyperplasia involving smooth muscle migration and proliferation, macrophage, and T lymphocyte infiltration (13). Intimal thickening as a result of this cellular infiltrate leads to the occlusion of
distal vessels first, presumably because of their smaller luminal area, and early post-transplantation, diffuse fibrous intimal thickening or vasculitis can occur (12). As these smaller vessels and branches are occluded, this can result in small, stellate infarcts (14). Lesions consisting of concentric intimal proliferation throughout distal and proximal vessels and throughout the entire coronary tree will eventually result (15). Despite such an aggressive allograft response, the internal elastic lamina remains almost intact and calcification rarely occurs or occurs very late (>6 years) post-transplantation (13). TVD affects the entire vasculature of the allograft, including venules (16, 17), donor aorta, donor pulmonary artery, and vasa vasorum of these large arteries (18). The media of the vessels are rarely thickened and may even become narrower than normal (19). Since the entire length of the vessel is affected, it is usually not possible to use angioplasty or coronary artery bypass grafting as a possible means of treatment (19). As the risks of infection have abated with clinical advances in treatment and the more effective management of severe early rejection with intensive induction immunosuppressive therapy during the perioperative period, and the advent of combined immunosuppressive therapy, chronic rejection has now emerged as an increasingly important cause of graft dysfunction and ultimate graft failure in long-term surviving transplant recipients (20, 21).
1.2.2 Organs Affected

Transplant vascular disease occurs not only in cardiac transplants but also in other solid organ transplants. One of the earliest documented cases of TVD was in the early 1950's by Hume and colleagues who observed vessels of renal allografts with marked luminal narrowing, some of which approaching complete luminal occlusion (22). Other organs affected by TVD include lung, hepatic, and pancreas allografts occurring with nearly equal frequency (15). Furthermore, virtually all of the features seen in coronary arteries of allograft hearts can also be seen in medium to large arteries of renal, hepatic, and pancreas allografts (23). TVD occurs in human renal (24, 25), hepatic (26, 27), lung (28, 29), and pancreas allografts (30). Cardiac allografts may be particularly susceptible to vascular changes and can be dramatically emphasized as the myocardium is dependent on coronary blood flow.

1.3 CAUSES AND CURRENT PREVENTION STRATEGIES OF TVD

1.3.1 Immune Factors

Although the exact pathogenesis of TVD is still not known, experimental and clinical evidence suggests that immunological factors augmented by non-immunological factors are involved in disease development. Within the clinical setting, the strongest evidence to support this is the fact that only the allograft vasculature is affected, sparing the recipient's vessels (31). It is suggested that early endothelial injury triggered by an immune response, followed by foam cell formation and accumulation of extracellular matrix and smooth muscle cell
proliferation, results in intimal thickening (32). Furthermore, clinical studies have shown that acute cellular rejection significantly correlates and is a predisposing risk factor of TVD (33-35). Experiments using rodent models of allograft vasculopathy have established the alloimmune response as paramount in the development of TVD, as vessel abnormalities were far more dramatic within allografts compared to syngrafts (36, 37). Moreover, native and host vessels are unaffected and if dosing is optimized, most immunosuppressive strategies can reduce or prevent intimal thickening (37). Histopathologic studies within rodent models (Lewis-to-F344) of allograft vasculopathy have demonstrated that arterial lesions develop in well defined stages with initial adherence of monocytes/macrophages and to a lesser degree, T lymphocytes to the endothelial lining and endothelitis (10). This is followed by intimal infiltration with monocytes, macrophages and T lymphocytes, followed by gradual accumulation of α-actin positive smooth muscle cells, resulting in progressive, diffuse, and concentric intimal thickening (38). In addition, NK cells and dendritic cells are also present within the arterial lesion (21). The immune cells up-regulate numerous inflammatory mediators including cell adhesion molecules, cytokines, chemokines, growth factors and macrophage activators as well as proteases that degrade the extracellular matrix (21).

In the transplant setting, antigens may be presented via indirect allore cognition and direct allore cognition, with the "indirect" route involving the uptake, processing, and presenting of graft antigens within the context of self-MHC on antigen presenting cells (APCs) to recipient T lymphocytes, whereas the
"direct" route involves the unique phenomenon in which recipient T lymphocytes recognize antigens presented on the donor APCs or "passenger leukocytes" such as dendritic cells, or even directly on the surface of donor endothelial cells (39). Both result in the stimulation and activation of host T-lymphocytes (40, 41).

Hyperacute rejection is sudden in onset and is often fatal. It can occur within minutes and even before closure of the incision (42). This type of vascular rejection occurs when there are circulating antibodies against the graft endothelium (43) resulting in the rapid necrosis of the graft within minutes to hours of graft revascularization (39). The targets of these preformed cytotoxic antibodies include HLA molecules and ABO antibodies (39). However, it remains to be seen whether anti-endothelial antibodies produced after allo-transplantation are directly cytotoxic, with the rare exceptions of anti-endothelial antibodies causing hyperacute or accelerated acute rejection (44). Fortunately, this type of vascular rejection has become less frequent with the pre-screening of transplant recipients for preformed cytotoxic anti-donor antibodies and ABO matching (31, 39, 45, 46).

Chronic rejection occurs as a result of both cellular and humoral mediated injury. T cell dependent humoral mechanisms are part of a cascade of events involved in the recognition and amplification of the allograft response against the transplanted organ with some studies reporting a correlation of anti-HLA antibodies with TVD (44). Antibodies against endothelial surface molecules such as MHC class I and ICAM-1 can result in endothelial and smooth muscle cell activation and the release of growth factors, exacerbating the inflammatory
response against the vessel wall (44). Similarly, antibodies against endothelial cells such as vimentin have been closely correlated with disease development and TVD (44), indicating that minor antigens released from damaged parenchymal cells within the graft may be responsible for a chronic antibody response (47). Although the number, duration and severity of acute rejections have all been reported to correlate with the risk of TVD development, the role of antibodies has not been universally confirmed (44).

1.3.2 Non-Immune Factors

There are several non-immunological risk factors involved that can contribute to the development of TVD such as age, obesity, hypertension, hyperlipidemia, smoking, diabetes, donor characteristics (48-51) and infections, particularly cytomegalovirus (52). The most consistent finding correlating to TVD was hyperlipidemia and insulin resistance, which is of particular importance, as insulin resistance and hypercholesterolemia occur in 50-80% of the heart transplant population (53). Post-transplant hyperlipidemia is a multifactorial event with obesity, cyclosporine level, cumulative dose of prednisone, and insulin resistance all contributing factors.

Ischemia and reperfusion of the graft at the time of transplantation is also one of the more important non-immune factors that may contribute to TVD. Early endothelial injury occurs even prior to ultrastructural detection, with a reduction of cellular function (54). Such injury can lead to the activation of the microvascular endothelium, resulting in the expression of adhesion molecules and cytokines.
and the release of reactive oxygen free radicals. This can induce subsequent activation of passing host leukocytes, monocytes and macrophages, and other aggressive mediators such as proteases, cytokines, and eicosanoids, further attracting host leukocytes (55). Ischemia and reperfusion injury can also lead to early oxidative stress of the allograft, and reperfusion of cardiomyocytes can lead to mitochondrial respiratory impairment and the generation of reactive oxygen species such as $\text{H}_2\text{O}_2$, $\text{O}_2^-$, and $\text{OH}^-$ (56). Such free radical mediated endothelial cell and myocyte injury can initiate the production of cytokines and further recruit inflammatory cells and enhance the inflammatory response (56).

1.3.3 Immunosuppressive Regimens of Transplant Recipients

Immunosuppressive strategies for cardiac transplant recipients can be divided into three major phases consisting of perioperative induction immunosuppression where the goal is to prevent rejection early after transplantation when it can be most severe, maintenance immunosuppression to induce “tolerance” and long-term graft survival, and rescue therapy for the treatment of transplant rejection (31). For over a decade after the first transplant surgeries, conventional immunosuppressive therapy consisted of steroids, azathioprine and antithymocyte globulin. However, their non-specific action on the immune system resulted in numerous severe side effects until the introduction of cyclosporine to the cardiac transplantation community in December 1980 at Stanford University (57, 58).
Cyclosporine A (CsA), also known as cyclosporine, cyclosporin, or ciclosporin, is a neutral, lipophilic, cyclic endecapeptide (MW 1203) extracted from *Tolypocladium inflatum Gams* (39). First purified by Jean-Francois Borel at Novartis (formerly known as Sandoz) in 1973 (59), its immunosuppressive effects on T-cell proliferation by ways of an anti-T helper cell mechanism was not discovered until a few years later (60, 61). For clinical use, CsA is stabilized with castor-oil (Cremophor) and olive-oil vehicles for intravenous and oral administration, respectively.

CsA is a prodrug and its immunosuppressive properties depend on the formation of a complex with the immunophilin cyclophilin, inhibiting calcineurin (CN) activity (62, 63). CsA enters the cell primarily through diffusion (64), and in lymphocytes blocks the transcription of cytokine genes IL-1, IL-2, IL-3, IL-4, IL-6, IL-7, TNF-α, and IFN-γ (62, 65-67), leading to the inhibition of helper T-cell proliferation (31, 68). During T-cell activation, APCs present foreign antigens to the T-cell receptor, activating the calcineurin pathway within the cytoplasm of the T-cell. CN is a calcium/calmodulin-dependent phosphatase which plays an integral part in the calcium-dependent signal generated after antigenic recognition by the T-cell receptor (62). CN dephosphorylates multiple molecules including nuclear factor of activated T-cells (NFAT), a multisubunit protein consisting of a cytollic fraction (NF-ATc) and a nuclear subunit (NF-ATn) (63, 69). Dephosphorylated NF-ATc translocates to the nucleus, where it binds to specific DNA sites in the promoter regions of cytokine genes, including IL-2, resulting in IL-2 gene transcription (64, 69). CsA is a CN inhibitor and acts by blocking the
calcium-activated CN pathway (64). It should also be noted that CsA does not completely block IL-2 production as a second signaling pathway, mediated through protein kinase C, also stimulates IL-2 production independent from the CN pathway and therefore resistant to CsA treatment (62, 63).

The advantage of calcineurin inhibitors is its specificity to the immune system, unaffecting other rapidly proliferating cells (70). Moreover, by impeding the production and release of IL-2 by T-helper cells and inhibiting interleukin-2 receptor (IL-2R) expression on both T-helper and T-cytotoxic cells, CsA consequently limits the differentiation and proliferation of cytotoxic T cells (59). CsA blocks the T-cell cycle at the G<sub>0</sub>-G<sub>1</sub> phase, limiting the activation of cytotoxic T-lymphocytes. However, this does not impair the ability of an activated cytotoxic T-cell to kill its target cells (62).

Unfortunately, administration of cyclosporine is associated with a range of serious side effects, among which nephrotoxicity is the most prominent and is dose-limiting (67, 71). Hypertension and hyperlipidemia also occurs in most patients (72). De novo diabetes mellitus also occurs in as many as 10% of patients at 1 year post-transplantation (64). CsA also causes neurological toxicity comprising of tremors, paresthesias, headaches, seizures, mental status changes, visual symptoms and insomnia (39, 64). Other side effects and toxicities include nausea, vomiting, cholestasis and cholelithiasis, hypertrichosis, gingival hyperplasia, hirsutism, and contributes to the development of osteoporosis (39, 64). As the incidence and severity of rejection decreases over
time post-transplantation, cyclosporine dosages can also be gradually titrated down.

In addition to a CN inhibitor such as CsA, cardiac transplant patients also undergo combination immunosuppressive therapy with an antiproliferative agent such as azathioprine (AZA) or mycophenolate mofetil (MMF), and steroids such as prednisone or prednisolone as part of the triple maintenance immunosuppressive therapy (73). AZA is a prodrug and a purine analogue that inhibits DNA and RNA synthesis by blocking the *de novo* and salvage pathways for purine biosynthesis (39, 64). When converted to its active metabolite 6-mercaptopurine in the liver, AZA is incorporated into the nucleic acids during mitosis and inhibits its synthesis and consequently, the proliferation of T- and B-lymphocytes (39, 64). MMF is a non-competitive inhibitor in the *de novo* synthesis of guanine nucleotides (64). Proliferating lymphocytes are dependent on this pathway due to the fact that it is the only pathway for their purine synthesis and DNA replication (64). This results in selective inhibition of lymphocyte proliferation by MMF (64). It has recently been shown that patients switching from AZA therapy to a combination therapy of MMF and prednisone delayed and even partially reversed luminal narrowing compared to baseline TVD at 1 and 2 years post-MMF administration (74).

Steroids were among the first immunosuppressive agents used in clinical transplantation and due to their potent immunosuppressive and anti-inflammatory properties are continued to be used today (64). They are lipid soluble compounds that diffuse freely across cell membranes, binding to high affinity
cytoplasmic glucocorticoid receptors and translocates to the nucleus, where it binds to a glucocorticoid response element within the DNA (39, 64). The receptor-steroid complex may also bind to other regulatory elements and inhibit their binding to DNA. Both result in transcriptional regulation and thereby altering the expression of genes involved in the immune and inflammatory response (64). Genes affected include those of cytokines, growth factors, CD40 ligand, GM-CSF, and adhesion and myosin heavy chain molecules (64). Corticosteroids also affect the number, distribution, and function of all types of leukocytes (64). In addition to their anti-inflammatory properties, corticosteroids are also directly toxic to lymphocytes (39). They also inhibit margination and chemotactic functions of lymphocytes, macrophages and neutrophils (39).

In the 1990's, tacrolimus (FK506) was introduced as an alternative to cyclosporine as a CN inhibitor in organ transplant recipients including cardiac transplant patients (73). Recently, sirolimus (rapamycin), everolimus (a derivative of rapamycin), and monoclonal anti-lymphocyte preparations of IL-2R antagonists have also been introduced into the clinical cardiac transplant setting, with the inclusion of rapamycin and everolimus leading to the reduction of TVD (73). However, despite the use of the triple drug immunosuppressive maintenance therapy (most commonly consisting of CsA, AZA, and prednisone) and minor improvements to the regimen, graft survival has not significantly increased over this past decade (51, 73).
1.4 PATHOGENESIS OF TVD

1.4.1 Role of Endothelial Cells

Endothelial cells line the luminal surface of blood vessels and rest on the sub-endothelial space, consisting of the basement membrane and extracellular matrix that extends to the internal elastic lamina. The vascular endothelium is a monolayer which lines all blood vessels throughout the entire body and constitutes approximately 1% of body mass with a total surface area of approximately 5,000 m² (75). Their normal life span in humans is approximately 30 years (76).

Endothelial cells form the interface between donor and recipient and are the first donor cells to be recognized by the host's immune system (77) and thus play a pivotal role in both acute and chronic rejection. Endothelial cells are the major determinate of vessel wall function. They normally inhibit thrombus formation, leukocyte adhesion, vascular smooth muscle proliferation, and regulate vasomotor tone (12, 78). Damage to the endothelial monolayer can result in impairment of endothelial function and predisposition to arterial inflammation, thrombosis, vasoconstriction, and vascular smooth muscle proliferation. As a result, endothelial injury and dysfunction are considered key events in the development of native and transplant associated allo-atherosclerosis. Endothelial injury can stimulate leukocyte infiltration, smooth muscle cell (SMC) migration from the media to intima, SMC proliferation, and foam cell formation (79-81). Endothelial cells are the primary target for the initiation of both cellular and humoral immunity (82). Continuous insults to the
endothelial barrier will result in a response to injury mechanism, leading to endothelial dysfunction (82). Furthermore, allograft endothelial injury leads to a proliferative intimal response and this cascade of events eventually leads to TVD (12).

Endothelial cells are exposed to insult during the initial ischemia and reperfusion of the transplant process, resulting in injury that can activate the microvascular endothelium. This can lead to the formation of oxygen free radical production and subsequent activation of circulating host leukocytes and macrophages (12, 83), resulting in an immediate inflammatory response and endothelial dysfunction. Ischemia / reperfusion (I/R) injury can also result in the expression of adhesion molecules, cytokines, growth factors, and the upregulation of MHC II expression by endothelial cells (77, 84-86). They also have immunological properties as antigen presenting cells and constitutively express MHC class I molecules (77). The expression of MHC class II has been described in every organ and is particularly striking on microvessels (77, 87, 88). Expression of MHC class II can initiate allograft rejection by activating CD4 T lymphocytes. Moreover, CD4 lymphocytes can cause upregulation of MHC class II antigens on endothelial cells (12). Damaged endothelial cells can also release non-HLA antigens such as vimentin (89), leading to a chronic antibody response, and subsequently contribute to TVD (44).
1.4.2 Role of Inflammatory Cells

Atherosclerosis is an inflammatory disease which results from a thickened lipid rich vascular intima (atheroma) and connective tissue (sclerosis). Russell Ross initially formulated the response-to-injury hypothesis of atherosclerosis from pathophysiologic observations in humans and animal models which initially proposed that endothelial denudation as the first step in atherosclerosis (90). He later updated this hypothesis with emphasis on endothelial dysfunction (81). Endothelial injury results in increased adhesiveness of the endothelial monolayer to leukocytes and platelets, and also leads to increased vascular permeability (81). Injury also induces the endothelium to have a procoagulant phenotype and the production of vasoactive molecules, cytokines, and growth factors (81). This can occur indefinitely, and as a result, the inflammatory response stimulates migration and proliferation of smooth muscle cells that become intermixed with the area of inflammation to form an intermediate lesion (81). Monocyte-derived macrophages and specific subtypes of T lymphocytes mediate this response at every stage of the disease (91, 92). These features are exacerbated within the allograft setting.

Initiation of the immune response can also occur as a result of presentation of donor antigen on the endothelium of allograft vessels by antigen presentation to recipient T lymphocytes (93). This can induce the production of cytokines, leading to cell division and proliferation of T lymphocytes (94). The cellular infiltrate of intimal proliferative lesions consist of modified smooth muscle cells, monocytes/macrophages, and T lymphocytes (19). Mononuclear
infiltration, often with an endothelialitis occluding the lumen, can occur (95). The close approximation of CD4 T cells and macrophages suggest that they interact at the site of the lesion (41). The interaction of T cells with graft endothelial cells not only initiates but also sustains the chronic immune response within the allograft (82). Depletion of CD4, but not CD8 T cells, prevented arterial lesion formation in an experimental model (96). Furthermore, eliminating the effects of CD4 T cells with the use of CD4 or MHC class II knockout mice resulted in reduction in intimal thickening by over 50% (41). Using anti-CD8 monoclonal therapy, TVD was abrogated in a swine heterotopic transplant model (97). Macrophages also play a key role in the disease process, as a lack of macrophages resulted in reduction of intimal thickening by two thirds and therefore, a greater effect than having no CD4 T cells (41). Although the exact immunological pathogenesis of TVD is still unknown, it is known that coronary artery endothelium has the ability to express both Class I and Class II antigens during the allograft response and may facilitate the immune response and contribute to TVD (77, 98). There is also a striking array of T lymphocytes and macrophages immediately underneath Class II positive endothelial cells in both early and late TVD, suggesting an ongoing immune response and the interaction between the cell types (99). As a consequence of the combined cellular and humoral immune response of the recipient, this may result in the upregulation of adhesion molecule expression and leukocyte migration into the allograft, exposure of collagen with increased endothelial thrombogenic activity, and the
production of growth factors and cytokines, eventually resulting in the formation of chronic allograft lesions, or TVD (82).

1.4.3 Permeability Related to TVD

The endothelial monolayer of blood vessels forms a barrier and regulates ingress and egress of blood constituents to the sub-endothelial space and the underlying tissues. Regulation of endothelial integrity and permeability are governed by several elements including: intercellular junctions, cell surface binding proteins, electrostatic charge of endothelial membranes, and composition of basement membrane (100). Evidence indicates that intercellular junctions, with the physical attachment between two contiguous cell membranes, are the most important of these (101, 102). The three known types of intercellular junctions identified between endothelial cells are tight junctions, gap junctions and adherens junctions. These junctions are formed by transmembrane proteins linked to both cytoplasmic and cytoskeletal proteins (101, 103, 104). To form the endothelial monolayer found within blood vessels, endothelial cells connect to one another by varying the number of tight junctions, or occluded zones, in which neighbouring endothelial cells limit the number of points of cell membrane contact or by apparent fusion in sections, or gap junctions, where cells form communicating junctions. In this fashion, discontinuous strands of tight junctions will link adjacent cells.

Several biological factors can modulate endothelial junctions and therefore, direct permeability across the endothelium. Inflammatory mediators
can increase endothelial permeability within minutes. Possible mechanisms of increased permeability include phosphorylation of proteins involved in the organization of endothelial junctions resulting in actin-myosin contraction. This results in a "centripetal" retraction of endothelial cells, and increased interendothelial gap formation. Paracellular transport of macromolecules through endothelial junctions is the major mechanism of increased endothelial permeability, in the absence of endothelial denudation, as a response to inflammatory mediators (102, 105).

1.5 RAT HETEROTOPIC CARDIAC TRANSPLANT MODEL

1.5.1 Abdominal Heterotopic Cardiac Transplantation Procedure

In the abdominal heterotopic cardiac transplant model, a donor heart is placed into the abdominal cavity of the recipient. This model was originally described by Ono and Lindsey in 1969 using a rat model (106) and is also applied in smaller rodents such as mice (107). In this model, the ascending aorta from the donor heart is sutured by end-to-side anastomosis to the abdominal aorta of the recipient. The donor pulmonary artery is anastomosed in the same manner to the inferior vena cava (IVC) of the recipient (Figure 3). The transplanted hearts behave functionally as an aorto-caval fistulae (107) and hemodynamically do not act as an active pump for the recipient. However, blood from the recipient enters the donor heart from the aorta via the recipient's abdominal aorta, and is diverted into the coronary arteries of the transplanted
heart via the closed aortic valve, resulting in perfusion of the graft myocardium. Upon perfusion of the myocardium, venous blood drains into the right atrium through the coronary sinus and is pumped into the recipient's IVC through the right ventricle (107).
Figure 3: Diagrammatic portrayal of the location and position of the heterotopically transplant heart, ligated vessels (x), and i.v. line for perfusion fixation.
1.5.2 Rationale for Selected Model

Numerous studies have documented the presence of TVD lesions in various animal models. One of the first documented cases was in a chronically rejecting canine cardiac allograft surviving at least 3 months, with diffuse fibrocellular intimal thickening and histologically resembling human allografts (108). Similar observations were also made in rabbit cardiac allografts, with significant lesion development within 2 weeks post-transplantation (109). Currently, many animal models are used to study TVD utilizing rat, mouse, rabbit, swine, and other animals undergoing aortic, orthotopic or heterotopic transplantation techniques. Unlike humans, experimental transplantation models provide controlled settings with stable microenvironments and dietary conditions. Among rat allograft models, aortic and heterotopic transplantation models are commonly used between either MHC match or MHC mismatch combinations. Although heterotopic cardiac transplantation may be more technically difficult to perform, it is advantageous over orthotopic aortic transplantation (110-112) in that both coronary arteries and myocardium can be examined in such a model (10, 113). When comparing rodent models of heterotopic cardiac transplantation, rats are more responsive to immunosuppressive therapies than mice, as the latter are relatively resistant to CsA and other immunosuppressants (114, 115). Our model consists of Lewis (LEW, RT-1\(^1\)) rats as donors and either a Lewis rat as the recipient for the syngraft condition or a Fisher (F344, RT-1\(^{1V}\)) rat as the recipient for the allograft condition. The LEW-to-F344 rat cardiac allograft model has been extensively studied by our laboratory (36, 116, 117) and
by numerous investigators (10, 113, 118-120). The Lewis and F344 strains share identical class I and class II antigens but differ in multiple non-MHC histocompatibility antigens (121). Therefore, rat cardiac allografts exchanged between MHC-matched and non-MHC disparate strains exhibit a continuous low-grade rejection process driven by non-MHC antigens, leading to the development of TVD. The advantage of the LEW-to-F344 heterotopic cardiac transplant model is that graft survival can occur in the absence of immunosuppressive therapy (10). This weak mismatch low responder strain combination has been proposed as a more suitable model to study transplant induced, delayed and progressive organ failure by many investigators (113, 122-125). Such recipients are subjected to a chronic rejection process resulting in the development of diffuse graft atherosclerotic lesions (113). Cyclosporine treatment in this model will result in the graft's indefinite survival (10, 113) and when given at lower doses, will exhibit only a modest reduction in the degree of vascular disease, and develop coronary artery disease similar to those seen in clinical graft atherosclerosis (10). Previous immunohistochemical studies have confirmed that end-stage lesions within the LEW-to-F344 models (i.e. Lewis allografts) are similar not only in appearance but in composition to human lesions, with an early mononuclear inflammatory stage and chronically rejecting Lewis grafts exhibiting intimal smooth muscle cell accumulation, and occasional monocytes and T-cells (10). Lewis and F344 rats are inbred strains that are genetically well defined, commercially available and, therefore, their use allows for highly reproducible experiments. Although this model has been well studied and is an established
model for cardiac allograft rejection, endothelial integrity has not been
classified in depth, particularly at early stages. Furthermore, ultrastructural
examination of allograft coronary arteries and aorta within experimental
animal models and humans are underrepresented within the literature.

1.5 HYPOTHESIS
Altered endothelial integrity, as reflected in structural changes, is present in the
rat allograft transplant model.

1.6 SPECIFIC AIMS

1. To examine the structural nature of endothelial integrity in the
coronary arteries of transplanted rats using en face silver nitrate
staining.
2. To examine, at the ultrastructural level, features of the
endothelium in the coronary artery and aorta of transplanted
rats using scanning electron microscopy (SEM).
3. To examine immune cell infiltration and disease progression, at
the ultrastructural level, by transmission electron microscopy
(TEM).

2 MATERIALS AND METHODS

Figure 4 diagrammatically illustrates the experimental design of the study.
Figure 4: Experimental design of study. Animal groups consist of male Lewis (LEW, RT1') non-operated rats and Lewis donor hearts into either Lewis recipients (syngrafts) or F344 (RT1'v') recipients (allografts). Treatment conditions for all groups consist of the immunosuppressant cyclosporine, vehicle (Cremophor EL) and saline. Transplanted animals were sacrificed at 1, 4, 21, and 42 days post-transplantation whereas non-transplanted controls were sacrificed at 4 days. Tissues were then harvested for silver nitrate staining and scanning and transmission electron microscopic examination.
2.1 MATERIALS

Male LEW (Lewis, RT-1\textsuperscript{1}) and F344 (Fisher, RT-1\textsuperscript{1v1}) inbred rats (225-250g) purchased from Charles River Laboratories (Montreal, PQ) were used for the entire study. Rats were housed under standard conditions with access to chow and water \textit{ad libitum} and acclimatized for 5 days upon arrival. The rats were divided into three groups: non-operated controls (LEW, n=6), donors (LEW, n=39) and recipients (LEW, n=18 or F-344, n=21). The recipients were further divided into two groups: syngraft group (n = 18, a LEW-to-LEW transplant model), and allograft group (n = 21, a LEW-to-F344 transplant model). These groups, along with the non-operated controls, were further randomly divided into 3 treatment groups: a saline-treated group, a vehicle-treated (Cremophor EL, Sigma Chemical Co, St. Louis, MO) group, and a cyclosporine-treated (Sandimmune I.V., Novartis Pharmaceuticals Canada, Dorval, PQ) group. All animals were injected subcutaneously with saline, cremophor EL, or cyclosporine (5 mg/kg/day) 2 days prior to surgery and up until time of sacrifice or a maximum 14 days post-transplantation. Cyclosporine treatment in such a manner would minimize the possibility of early acute rejection of the allograft (113). Cyclosporine injection at our experimental dose results in approximately 1000ng/mL of cyclosporine in the blood after 14 days of treatment (126). In the absence of an adverse lipid profile, the coronary arteries of the Lewis-to-F344 model develop arterial intimal lesions similar to those seen in human transplant vessels (113). The study was approved by the Animal Care Committee of the University of British Columbia.
2.2 RAT HETEROTOPIC CARDIAC TRANSPLANTATION

Standard heterotopic abdominal heart transplant procedures were performed as previously described (36, 106, 117). Anesthesia was induced with 4% halothane and maintained with 1-2% halothane (Halocarbon Laboratories, River Edge, NJ) for both donors and recipients. Donor rats were infused with heparinized saline. The superior vena cava, inferior vena cava, and pulmonary veins were ligated and the pulmonary artery and the aorta transected. The explanted heart was then rinsed and stored in ice-cold heparinized saline. Recipient rats were anesthetized as described above, and their abdominal aorta and inferior vena cava clamped. The aorta and pulmonary artery of the donor heart was then anastomosed to the recipient's abdominal aorta and IVC, respectively, in an end-to-side manner. Implantation into the recipient was completed within 30-40 minutes of removal of the donor heart. GELFOAM® (Pharmacia & Upjohn, Mississauga, ON) was used as a haemostatic to seal any leaks around the points of anastomosis. Upon surgery, one dose of buprenorphine (Buprenex® Injectable, Reckitt & Colman Pharmaceuticals, Richmond, VA) (0.01mg/kg i.m.) was administered as an analgesic.
2.3 TISSUE COLLECTION

At 1, 4, 21 and 42 days post-transplantation, rats were anaesthetized (as above) and perfusion fixed by gravity infusion with paraformaldehyde and glutaraldehyde. Briefly, three intravenous (i.v.) bags were filled with 100mL each of heparinized saline, modified Karnovsky fixative (1% paraformaldehyde / 1.25% glutaraldehyde), or 50mL of Karnovsky fixative (2% paraformaldehyde / 2.5% glutaraldehyde). A catheter (23 gauge) was placed into the abdominal aorta and used to administer the above solutions. Each i.v. bag was placed 100 cm above the rat and, therefore, constant pressure and flow was maintained throughout the procedure. Perfusion began with heparinized saline, followed by modified Karnovsky fixative, each for 20 minutes, and ending with Karnovsky fixative for 10 minutes. Therefore, a constant rate of 5mL/min was achieved for all perfusion solutions. Confirmation of fixation was done both visually and by palpation of various organs within the rat. Upon confirmation of fixation, both native and transplanted hearts were removed. Portions of the aorta, right epicardial coronary artery, and ventricular septal artery were removed and harvested for SEM and TEM. The remainder of the heart was used for en face silver nitrate staining.

To minimize damage to the tissue and particularly to the endothelium for SEM, coronary arteries were cut in half longitudinally upon dissection from the heart. With this technique, mechanical damage from harvesting was observed principally along cut edges and were easily identified. Moreover, SEM sampling and observations on tissue were performed from the mid-regions of each vessel
and not along the cut edges. Occasionally, mechanical damage occurred during the mounting procedure of the vessels onto stubs (after critical point drying), prior to sputter coating with gold palladium. As such damage occurred post-fixation, it can be easily identified as apparent crushing or breaking of cells and vessel walls and was easily distinguished from biological abnormalities observed within the endothelium.

2.4 EN FACE SILVER NITRATE STAINING

Staining of endothelial cell junctions was performed based on an established method (127). Briefly, coronary arteries and aorta were perfused with 5 mL each of ddH₂O, 0.05% AgNO₃, ddH₂O, 1%NH₄Br/3% CoBr, and ddH₂O (in that order) at a rate of 5mL/min (Figure 5). Vessels were then removed from the myocardium, cut longitudinally, and placed overnight in Karnovsky’s fixative under a white fluorescent lamp, allowing for oxidation of AgCl along endothelial cell borders and basal lamina (127). The silver nitrate stained vessels were then mounted en face on glass slides with glass coverslips using 25% glycerol as mounting medium. Specimens were analyzed by light microscopy (Nikon Eclipse E600, Nikon Canada Inc., Mississauga, ON) and images captured digitally (Spot, Diagnostic Instruments Inc., Sterling Heights, MI) with appropriate software (Spot Version 3.4.2 for Windows, Diagnostic Instruments Inc., Sterling Heights, MI).
**Figure 5:** Diagrammatic and pictorial portrayal of the silver nitrate perfusion procedure conducted for the study. $X =$ ligation.
2.5 SCANNING ELECTRON MICROSCOPY

Silver nitrate staining of the endothelium of allografts suggested a compromise in endothelial integrity as a result of injury within non-immunosuppressed allografts. To further examine and determine the nature of such endothelial abnormalities ultrastructurally, SEM was performed on representative samples for each treatment and time point.

Upon removal from the heart (refer to section 2.3), vessels were stored in Karnovsky's fixative at 4°C for no longer than 24 hours. Following three washes in 0.1M cacodylate buffer (pH 7.4), tissues were post-fixed in 1% osmium tetroxide and 1% potassium ferrocyanate in 0.1M cacodylate buffer (pH 7.4). Vessels were then dehydrated through graded isopropyl alcohol, transferred to amyl acetate, and critical point dried in CO₂. The vessels were then mounted on aluminum stubs with adhesive spots and sputter coated with gold (Nanotech SEM Prep 2 apparatus, Nanotech, Manchester, UK). Specimens were viewed in an SEM (Cambridge T150 stereoscan microscope, Cambridge Instruments, Cambridge, UK) at 20 kV to evaluate the integrity of the endothelial cells and their borders, along with the presence of leukocytes and platelets. Endothelial topography and integrity was documented photographically.

The SEM observations for this study were from two to three pieces of aorta, three pieces of right epicardial coronary artery, and three pieces of ventricular septal artery from each transplanted or native heart. Observations of control endothelium were from three non-transplanted rat hearts. Observations of saline treated allograft hearts at 1, 4, 21 and 42 days post-transplantation were
from one, three, two and three Lewis rat hearts, respectively. Observations of vehicle treated allograft hearts at 1, 4, 21 and 42 days post-transplantation were from one, one, one and two Lewis rat hearts, respectively. Observations of cyclosporine-treated allograft hearts at 1, 4, 21 and 42 days post-transplantation were from one, one, one and two Lewis hearts, respectively. Two to three pieces of aorta from each heart were viewed using the SEM. The total areas of aorta examined for saline-treated, vehicle-treated, cyclosporine-treated transplants and non-transplanted controls were 31.8, 7.7, 22.8 and 31.8 mm\(^2\), respectively. All right coronary and septal artery pieces from each heart were examined over their entire length using the SEM. If intact endothelium was absent, the specimen was examined closely to characterize the surface, which included platelets, basal lamina, elastic lamina, or other cell types. Any abnormalities in endothelial integrity or topography were documented photographically. Due to the small diameters and curvature of coronary arteries, we have reported lengths rather than the areas examined. For non-transplanted hearts, approximately 49 mm of right coronary artery and 44 mm of the septal artery were examined. A total of approximately 45 mm and 31 mm (saline-treated), 30 mm and 29 mm (vehicle-treated), and 19 mm and 15 mm (cyclosporine-treated) from right coronary artery and septal artery, respectively, from rats sacrificed at 1, 4, 21 and 42 days post-transplantation were examined.
Sections of coronary arteries from saline treated and cyclosporine treated control and allograft rat hearts were examined by TEM. Following dehydration, sections of coronary arteries were infiltrated and embedded in Epon 812, thin sectioned and stained with lead and uranium salts. Observation was performed with a digital TEM (Hitachi H7600 120keV PC-TEM, Chiyoda-ku, Tokyo) and images captured digitally.

Selected specimens of aorta from saline treated allografts were also processed for TEM in order to determine the identity of leukocytes observed at 21 days post-transplantation by SEM. This was the only time point and treatment for which SEM examinations showed multiple amoeboid shaped leukocytes adherent and polarized on the endothelial surface of the aorta. Following dehydration, infiltration and embedding as described above, observations were performed by a conventional TEM (Philips EM400, Eindhoven, the Netherlands) and documented photographically.
3 RESULTS

To address specific Aim 1, control, syngraft, allograft and their respective native coronary arteries and aorta were initially examined at the light microscopic level utilizing en face silver nitrate staining to observe endothelial pavement patterns within saline-, vehicle- and CsA-treated animals. Any endothelial abnormalities such as the presence of interendothelial gaps or areas of missing cells were documented digitally. Upon examination at the light microscopy level, coronary arteries and aorta were further investigated by electron microscopy using SEM and TEM. To address specific Aim 2, SEM was used to analyze endothelial topography. With SEM, we are able to observe the luminal endothelial surface ultrastructurally and complement our observations on silver nitrate stained material. Areas of injured and denuded endothelium and exposed basal lamina and elastic lamina can be further analyzed along with leukocyte and platelet presence. Upon analysis by SEM and to address specific Aim 3, we examined arteries by TEM to further characterize endothelial integrity and injury along with any ultrastructural cellular activity and distinctions. We also used TEM to characterize leukocytes that were observed by SEM and reflected early disease progression. TEM can also be used to examine early leukocyte presence and transmigration prior to detection by light microscopy and is therefore a very useful tool to further enhance and complement our light microscopic and SEM observations.
3.1 SILVER NITRATE STAINING

3.1.1 Control Non-Transplanted Animals

Control non-transplanted hearts all exhibited normal looking endothelial cell borders, with cobble stone pavement or chicken wire-like patterns as demarcated by silver nitrate staining of endothelial cell borders. This pattern was seen in saline treated, vehicle treated, and cyclosporine treated animals (Figure 6a-c). There was no clear visible difference in endothelial pavement patterns between the three treatment conditions among control vessels.

3.1.2 Syngraft Transplanted Animals

Syngeneically transplanted animals were examined at 1, 4, 21, and 42 days post-transplantation (Figure 6d-g). Syngraft Lewis hearts at 1 day post-transplantation exhibited some endothelial damage in coronary arteries in the form of interendothelial gaps (Figure 6d). This can be observed as the presence of "spots" of silver precipitate between neighbouring endothelial cells or regions containing long lines of silver precipitate and thicker than those designated as cell borders. At 1 and 4 day post-transplantation within syngrafts, large regions of missing endothelial cells and exposed basal lamina were absent (Figure 6d,e). At 4 days post-transplantation, endothelial damage was primarily in the form of interendothelial gaps, without areas of exposed basal lamina and regions of missing endothelial cells (Figure 6e). At 21 days post-transplantation, the endothelial monolayer was intact with well-defined cell borders and very few interendothelial gaps present (Figure 6f). Regions of missing cells and exposed
basal lamina were absent. At 42 days post-transplantation, endothelial pavement patterns were similar to those seen at 21 days post-transplantation with very few interendothelial gaps and the absence of missing endothelial cells (Figure 6g).
Figure 6: Silver nitrate stained coronary arteries, mounted *en face*, of non-transplanted control animals (Panels a-c) and syngraft non-treated animals (Panels d-g). Panel a. No treatment compared to (Panel b) vehicle and (Panel c) cyclosporine treatment. Note all vessels have well defined flagstone-like pattern around endothelial cell borders representing an intact endothelial layer. Panels d and e. At 1 and 4 days post-transplantation, syngraft coronary arteries exhibited some interendothelial gaps (arrows). Panels f and g. At 21 and 42 days post-transplantation, coronary arteries remain intact with well-defined cell borders and minimal interendothelial gaps present. Scale bar = 50 μm.
3.1.3 Saline-Treated Allograft Animals

Saline-treated Lewis hearts transplanted into F344 hosts exhibited endothelial injury as early as 1 day post-transplantation with the formation of interendothelial gaps and regions of missing cells. This resulted in exposed basal lamina, as demarcated by areas of darkly stained silver nitrate (Figure 7a). At 4 days post-transplantation, there was an apparent increase in the presence of endothelial gaps as well as an increase in areas of missing endothelial cells compared to 1 day post-transplantation (Figure 7b). At 21 days post-transplantation, there was an increase in the presence of interendothelial gaps and areas of missing cells (Figure 7c). At 42 days post-transplantation, there was a dramatic increase in the presence of missing endothelial cells, with large areas of exposed basal lamina and extracellular matrix, as signified by large regions of silver nitrate staining (Figure 7d).

3.1.4 Cyclosporine-Treated Allograft Animals

All cyclosporine-treated animals, regardless of time points, were treated for 2 days prior to surgery and for up to 14 days post-transplantation. At 1 day post-transplantation, endothelial integrity was preserved with very little interendothelial gaps present and very few areas of missing endothelial cells and exposed basal lamina (Figure 7e). Similarly, at 4 and 21 days post-transplantation, the endothelium remained intact without a significant increase in the presence of interendothelial gaps or missing cells (Figure 7f,g). However, at 42 days post-transplantation, there was a gradual decrease in the integrity of the
Figure 7
Figure 7: Silver nitrate stained coronary arteries from saline-treated (Panels a-d) and cyclosporine treated (Panels e-h) allograft hearts. Panel a. At 1 day post-transplantation, allograft coronary arteries already exhibited areas of missing cells (stars) as well as the presence of interendothelial gaps (arrow) represented by the silver nitrate staining. Panel b. At 4 days post-transplantation, damage to the endothelium was also present with the presence of gaps and missing cells. Panel c. At 21 days post-transplantation, there was an increase in regions of missing cells and gaps. Panel d. By 42 days post-transplantation, large regions of missing endothelial cells and subsequent exposure of basal lamina can be seen with large areas of silver nitrate stained tissue designating regions of missing endothelial cells and exposed basal lamina. Note the severely damaged endothelial layer with numerous missing cells (stars). Panels e and f. Cyclosporine treatment prevented abnormalities and protected the endothelium, resulting in preserved endothelial integrity at both 1 (Panel e) and 4 (Panel f) days post-transplantation. Panels g and h. At 21 (Panel g) and 42 (Panel h) days post-transplantation, the protective effects of cyclosporine were limited and endothelial integrity gradually decreased with a gradual increasing presence of interendothelial gaps. However, large areas missing cells were absent. Scale bar = 50 μm.
endothelium with the appearance of interendothelial gaps (Figure 7h). Damage
to the endothelium was limited to these gaps as large areas of missing
endothelial cells and exposed basal lamina were not present (Figure 7h).

3.2 SCANNING ELECTRON MICROSCOPY

3.2.1 Control Non-Transplanted Animals

Normal endothelial topography from control rat aorta, right epicardial
coronary artery, and ventricular septal artery was observed (Figure 8a,b,c).
Endothelial cell shapes, defined and visualized by cell borders, varied from
axially elongated and polygonal in the aortic root to long and spindle shaped in
the right coronary and ventricular septal arteries. Intercellular borders were intact
with no evidence of intercellular gaps. Variable numbers of small microvilli were
evident over the endothelial cell surface of these vessels (Figure 8a,b,c). The
endothelial cell surface of the aorta appeared topographically flat, whereas the
coronary arteries exhibited longitudinal folding along the cell surface, which was
most pronounced in the smaller septal arteries. Endothelial abnormalities such
as interendothelial gaps, or bare regions of missing cells, were absent in all
control vessels examined. In addition, neither leukocytes nor platelets were
observed adherent in control vessels.
Figure 8: Scanning electron microscopy of normal endothelial surface of (Panel a) rat aorta, (Panel b) right epicardial coronary artery and (Panel c) ventricular septal artery. Endothelial cell shapes vary from polygonal in the aorta (Panel a) to long and spindle shaped in the right epicardial (Panel b) and ventricular septal (Panel c) coronary arteries. Cell borders are visible (arrow) and intact without intercellular gaps. Small microvilli are scattered over the endothelial surface of all three arteries (arrowhead). Longitudinal folding is most pronounced in the smaller septal arteries.
3.2.2 Non-Immunosuppressed Allograft Animals

At 1 and 4 days post-transplantation, changes in endothelial topography and integrity were observed in both saline-treated and vehicle-treated allograft hearts (Figure 9a-h). In addition, adherent and transmigrating leukocytes were observed on large areas of the aortic endothelium (Figure 9a). Numerous small intercellular gaps or holes were present between leukocyte covered endothelial cells (Figure 9b). Endothelial cells appeared to be exfoliating from their subtending extracellular matrix (Figure 9c), exposing the underlying basal lamina and elastic lamina (Figure 9d). For this study, a continuous sheet of extracellular matrix material represents basal lamina, whereas an anastomosing layer with fenestrations represents elastic lamina (Figure 9e). Platelets in various stages of adhesion, activation, and spreading were observed on large areas of exposed extracellular matrix (Figure 9f,g,h). Furthermore, small clusters of adherent leukocytes and occasional intercellular gaps were observed between endothelial cells of coronary arteries of saline-treated allograft hearts at 1 and 4 days post-transplantation, confirming our observations using silver nitrate (Figure 10a,b).
Figure 9
Figure 9: Scanning electron microscopy of allograft hearts at 1 and 4 days post-transplantation. Panels a and b. Areas of intact endothelium were covered with adherent and polarized electron bright leukocytes (a, b, arrow). There were also large areas of bare basal lamina (stars) as a result of endothelial exfoliation and the presence of interendothelial gaps (b, arrowheads). Panels c and d. Regions of endothelial cells peeling off (arrow), resulting in exposure of basal lamina below (star). Panel e. Higher power view of elastic lamina and fibers as a result of endothelial exfoliation (arrow). Panel f. Various areas were almost completely covered with platelets adherent to regions of bare extracellular matrix. Panel g. Platelets in progressive stages of adhesion, activation, and spreading (arrows). Panel h. Spreading platelets exhibited flattening and long peripheral cellular extensions (arrow).
Figure 10: Scanning electron microscopy of allograft hearts at 1 (Panel a) and 4 (Panel b) days post-transplantation. Panel a. At 1 day post-transplantation, coronary artery endothelium contained numerous gaps (arrows). Panel b. At 4 days post transplantation, polarized leukocytes (arrows) were present near inter-endothelial gaps (arrowheads).
At 21 and 42 days post-transplantation, dramatic changes in both endothelial topography and cell populations were observed. Large areas of the endothelial surface of both saline-treated and vehicle-treated allograft aortas were covered by a dense population of giant cells with numerous microvilli and ruffled morphology (Figure 11a,b,c). Smaller leukocytes were infrequently observed between these giant cells (Figure 11a). Although most endothelial cell borders remained intact (Figure 11c), occasional large interendothelial gaps revealed leukocytes within the subendothelial space. In addition, some endothelial cells had lost microvilli and developed perinuclear transendothelial fenestrae, where basal lamina was visible through these fenestrae (Figure 11d).

Leukocytes were also observed undergoing 4 stages of diapedesis; tethering, adhesion, penetration or transmigration, and end-stage diapedesis (Figure 12a-d). Initially, a leukocyte can be seen tethered to the endothelial cell surface with elongation in the direction of blood flow (Figure 12a). Upon initial adhesion and tethering, leukocytes can then adhere firmly to the endothelial cell surface and form lamellipodia and begin the process of transmigration into the subendothelial space (Figure 12b). An adherent leukocyte was observed in the process of transmigration into the subendothelial space at a tricellular corner of aortic endothelial cell borders (Figure 12c). End stage diapedesis, in which a transmigrated leukocyte can be seen underneath aortic endothelium, is visible topographically as a result of electron bright mounds or “raised elevation” on the surface of the endothelium (Figure 12d).
Figure 11: Scanning electron microscopy of 21 and 42 day allograft aortic intimal disruption. Panels a and b. Amoeboid giant cells (arrowheads) and leukocytes (thin arrow) were present on allograft endothelium with intercellular gaps (thick arrows). Panel c. Leukocytes were also present in regions where endothelial cell borders remained intact (arrow) with the presence of giant cells (arrowheads). Panel d. Endothelial cells also exhibited transcellular fenestra around the nucleus (arrows).
Figure 12: Scanning electron microscopy of leukocytes undergoing 4 stages of diapedesis. Panel a. On portions of endothelium, leukocytes were observed to be tethered and polarized in the direction of blood flow on the endothelial cell surface. Panel b. Leukocyte adherent to the endothelial cell surface. Panel c. Leukocyte in the process of transmigration into the subendothelial space. Panel d. End stage diapedesis where a leukocyte has penetrated into the subendothelial space, as indicated by electron bright mounds and raised elevation (arrow).
3.2.3 Cyclosporine-Treated Allograft Animals

A minimal amount of endothelial cell exfoliation was observed in aortas at 1 and 4 days post-transplantation in cyclosporine-treated allografts (Figure 13a). There were limited numbers of adherent and transmigrating leukocytes and few intercellular gaps present (Figure 13a). Within coronary arteries, there were limited numbers of adherent or polarized leukocytes, but intercellular gaps were absent (Figure 13b).

By 21 and 42 days post-transplantation, endothelial abnormalities and leukocyte adhesion and migration were evident within cyclosporine-treated allografts. Accumulations of adherent leukocytes were observed on the endothelial cell surface and in the intima, although giant cells were absent (Figure 13c,d). Even thought SEM is normally restricted to the visualization of tissue topography and therefore the endothelial cell surface, viewing the intima and leukocytes within the subendothelial space was possible in some of our samples due to fortuitous post-fixation artifacts such as crushing and breaking of the tissue surface and cells, resulting in the formation of cracks within the endothelium, and exposure of the subendothelial space (Figure 13d). Furthermore, intercellular apertures were observed at tricellular corners in the endothelium among adherent leukocytes (Figure 13e). In coronary arteries, adherent leukocytes and gaps were observed on cessation of cyclosporine treatment at 21 and 42 days post-transplantation (Figure 13f).
Figure 13: Scanning electron microscopy of endothelial surfaces of cyclosporine-treated allograft aorta and coronary arteries. Panel a. Areas of endothelial cell exfoliation (arrowheads) with adherent platelets in an allograft aorta at 4 days post-transplantation. An adherent, transmigrating leukocyte is also shown (arrow). Panel b. At 21 days post-transplantation, adherent and polarized leukocytes (arrows) were also observed on coronary artery endothelium. Panel c. Leukocytes were adherent on aortic endothelium (arrows) neighbouring endothelial intercellular gaps (arrowheads). Panel d. Artificially fractured endothelium revealed leukocytes within the subendothelial space (arrows). This view would normally be obscured in SEM. Panel e. Intercellular apertures were present at tricellular corners in the aortic endothelium (arrow). Lines are drawn to indicate cell borders of endothelial cells 1, 2 and 3. Panel f. Within a 21 day coronary artery, adherent leukocytes (arrows) and intercellular gaps (arrowheads) were observed.
3.2.4 Processing Artifacts Involved in the SEM Procedure

Three different processing artifacts were identified in the samples used for our study. Abrasions of the endothelium produced from handling of tissues following critical point drying were the easiest to identify. Tissues in these areas resulted in topographical details that were crushed, cracked, and torn (Figure 14a). The second processing artifact observed were large endothelial blisters (Figure 14a,b,c). These blisters were eliminated by insuring osmification occurred immediately following glutaraldehyde fixation. The third type of artifact observed were uniformly round microblisters with electron bright edges, which we were not able to eliminate (Figure 14d).
Figure 14: Endothelial blistering was observed as artifacts involved in the SEM procedure. Distensions of the endothelial surface into the vascular lumen were observed in all vessel types including septal artery (Panel a), right coronary artery (Panel b), and aorta (Panel c). Panel b. Some blisters appeared as if they had burst and collapsed (arrows). Panels c and d. Small endothelial perforations were also observed in the aorta and coronary vessels (arrow).
3.3 TRANSMISSION ELECTRON MICROSCOPY

3.3.1 Control Non-Transplanted Animals

Control non-transplanted coronary arteries exhibited normal looking uninterrupted endothelium, with no evidence of endothelial discontinuity (Figure 15a). Spindle shaped endothelial cells varied from approximately round to elongated depending upon the plane of section. The internal elastic lamina was observed underneath the endothelium (Figure 15a,b). The layer of the vessel wall known as the tunica intima consists of the endothelial cells, basal lamina and internal elastic lamina (Figure 15a). Some endothelial cells were in contact with the internal elastic lamina through apertures in the basal lamina (Figure 15b). The internal elastic lamina separates the intima from the next layer of the vessel wall, the tunica media, of which within these coronary arteries was about two smooth muscle cell layers thick with some elastic fibers present between them. The adluminal surface of endothelial cells exhibited thin cytoplasmic projections with an average length of 500nm (Figure 15b). Smooth muscle cells contained dense bodies that were both membrane associated and cytoplasmic with a basal lamina surrounding their cell membranes (Figure 15b). In addition, there was evidence of metabolic activity within profiles of endothelial and smooth muscle cells, as indicated by the presence of numerous RER and mitochondria.
Figure 15: Transmission electron microscopy of control non-operated coronary arteries. Panel a. Control coronary arteries exhibited normal looking endothelium with an intact endothelial monolayer and many points of cell-to-cell contact, with the internal elastic lamina (asterisks) below and smooth muscle cells and some collagen within the media. I = Tunica Intima, M = Tunica Media. Scale bar = 2 μm. Panel b. Higher power image of the surface of endothelial cells with small cytoplasmic projections protruding towards the vascular lumen (thick arrow). Endothelial cells can also be seen to have close contact with the internal elastic lamina (arrows). Scale bar = 500 nm.
3.3.2 Saline-Treated Allograft Animals

At 1 day post-transplantation, endothelial cells within saline-treated allograft hearts exhibited ruffled cell morphology on the luminal surface with numerous cytoplasmic extensions. Some endothelial cells contained abundant RER and numerous mitochondria, suggesting increased cellular metabolism (Figure 16a,b). On the adluminal cell surface, endothelial cells had numerous caveolae (Figure 16a,b). Also present within endothelial cells were numerous vacuoles which were most prominent at cell margins adjacent to intercellular junctions (Figure 16c). Apparent endothelial desquamation left gaps through which extracellular matrix was exposed at 1 day post-transplantation (Figure 16d). Although there were regions where the internal elastic lamina was exposed to the vascular lumen as a result of endothelial denudation, the internal elastic lamina remained intact and continuous throughout the vessel, and smooth muscle cells below exhibited unaltered ultrastructure and organization (Figure 17a,b). Smooth muscle cells within the media were densely packed with little intercellular space and formed a multi-cellular layer. From a combination of longitudinal and cross sections, it is clear that the smooth muscle cells aligned circumferentially around the arteries (Figures 17a,b). In contrast to smooth muscle cells, endothelial cells had altered surface topography on the luminal surface exhibiting a “bursting” like morphology and cytoplasmic extensions resulting in the focal increases in endothelial and therefore, luminal surface area (Figure 17b,c). At 1 day post-transplantation, monocytes that have already completed diapedesis were observed underneath endothelial cells (Figure 17b).
Figure 16: Transmission electron microscopy of 1 day allograft coronary arteries exhibiting endothelial cell activity, vacuolization, denudation, and exposure of elastic lamina. Panels a and b. Endothelial cells exhibiting cellular activity as indicated with the presence of numerous caveolae (arrowheads), mitochondria (stars), ribosomes and rough endoplasmic reticulum (arrows). Scale bar = 500 nm. Panel c. Vacuoles were present within endothelial cells. Scale bar = 500 nm. Panel d. Regions in which the internal elastic lamina was exposed to the vascular lumen as a result of interendothelial gap formation (thick arrow). Scale bars = 500 nm.
Figure 17: Transmission electron microscopy of 1 day allograft coronary arteries exhibiting endothelial cytoplasmic extensions and immune cell infiltration. Panel a. Endothelial vacuolization (arrows) has occurred. An intact internal elastic lamina is present below. Scale bar = 10 μm. Panel b. The surface of the endothelium had cytoplasmic extensions protruding towards the lumen. A monocyte can be seen migrated within the subendothelial space and in contact within the internal elastic lamina. Scale bar = 2 μm. Panel c. Higher power reveals disruptions to the cell membrane and cytoplasm of the endothelial cell surface, all towards the luminal surface, exhibiting a "bursting" like morphology. Panel d. A neutrophil that has completed diapedesis at an interendothelial cell border with adjacent endothelial cell borders wrapping around the neutrophil. This neutrophil can be seen extending and contacting the basal lamina and elastin fibers underneath the endothelium (arrows). Scale bar = 2 μm.
At higher power (18,900 X), a neutrophil can be seen extending and contacting the basal lamina and elastin fibers underneath the endothelium (Figure 17d). Some monocytes have already migrated into the sub-endothelial space and within the internal elastic lamina (Figure 17b).

At 4 days post-transplantation, further evidence of endothelial cell exfoliation was observed like the mostly detached and necrotic endothelial cell (Figure 18a). However, the internal elastic lamina remained intact (Figure 18c). As observed at 1 day post-transplantation, vacuoles within endothelial cells were also present at cell margins adjacent to intercellular junctions (Figure 18b). Smooth muscle cells within the media were unaltered morphologically, with cells closely adjacent to each other (Figure 18c).

At 21 days post-transplantation, both resting and activated platelets were observed to be adherent to the endothelial cell surface (Figure 19). Activated platelets were identified by their long globular cytoplasmic extensions as well as their homogeneous cytoplasm lacking in organelles, granules and glycogen particles. Importantly, this was the first time point in which intimal thickening could be detected with the presence of monocytes within the neointima (Figure 19). In addition, large vacuoles slightly larger than 1μ in diameter were frequently observed at endothelial cell borders (Figure 20a). The luminal surface of endothelial cells were ruffled with various cytoplasmic extensions that ranged from approximately 100 nm on the surface (Figure 20b) to over 500nm protruding from the surface of the endothelial cells at intercellular borders and marginal flaps (Figure 20c,d).
Figure 18: Transmission electron microscopy of 4 day allograft coronary arteries exhibiting evidence of endothelial cell exfoliation and vacuole formation. Panel a. Endothelial cell (arrow) can be seen exfoliated and adherent to a mononuclear cell (star). Scale bar = 2 μm. Panel b. Vacuoles were present within endothelial cells and prominent at cell-to-cell borders. Scale bar = 500 nm. Panel c. The internal elastic lamina remains intact throughout the vessel. Smooth muscle cells within the media were observed to have normal looking morphology and cells arranged closely together. Scale bar = 2 μm.
Figure 19: Transmission electron microscopy of a 21 day allograft coronary artery with platelets adherent to the endothelium. Homogeneous area of cytoplasm within the activated platelet suggests a degranulated platelet (thick arrow) whereas the neighbouring adherent platelet still contains granules (arrowhead) with possible glycogen particles (thin arrows). Monocytes (stars) were also present within the newly formed neointima. Scale bar = 2 µm.
Figure 20: Transmission electron microscopy of 21 day allograft coronary arteries with endothelial abnormalities of vacuole formation and small cytoplasmic extensions at interendothelial junctions. Panel a. Endothelial cells can be seen to have large, empty vacuole-like structures at slightly larger than 1µ in diameter at cell-to-cell borders. Scale bar = 500 nm. Panels b, c, d. The surface of endothelial cells also have a ruffled morphology with various cytoplasmic extensions ranging from approximately 100 nm on the surface (Panel b) to those over 500nm protruding on top of neighbouring endothelial cells (Panel c) and interdigitating at endothelial junctions (Panel d). Scale bars = 500 nm.
Although the media of the allograft appeared to be relatively unaffected at previous time points, by 21 days post-transplantation, disruptions were observed. The density of smooth muscle cells within the media decreased significantly and interstitial space appeared to increase (Figure 21a). Furthermore, smooth muscle cell shape and organization was altered within the media (Figure 21a) and contained numerous vacuoles within their cytoplasm in addition to their large contractile apparatus (Figure 21b). Within the media, immune cells including monocytes and macrophages as well as collagen became prominent constituents of the interstitium (Figure 22a,b). Monocytes were distinguished by their smaller cell size relative to macrophages and ultrastructurally have an oval-, kidney- or horseshoe-shaped nucleus eccentrically located within the cell, and cytoplasm containing very fine azurophilic granules (Figure 22a). Macrophages were larger in size, also identified with an oval or kidney-shaped nucleus eccentrically located, with cytoplasm containing numerous secondary lysosomes (Figure 22b,c,d). In the process of monocyte-to-macrophage transformation, there is an increase in protein synthesis as well as cell size. Importantly, when adequately stimulated, macrophages can increase in size, forming epithelioid cells, or several may fuse together to from multinuclear giant cells (see later) found in pathologic conditions.
Figure 21: Transmission electron microscopy of a 21 day allograft coronary artery with disruptions within the media. Panel a. Low magnification image of a coronary artery with large areas of intercellular space (arrows) between smooth muscle cells. Within the media, an elongated smooth muscle cell (star) is present near the internal elastic lamina and a macrophage (arrowhead) present within the deep media. Scale bar = 10 μm. Panel b. Smooth muscle cell with numerous vacuoles within the deep media. Scale bar = 2 μm.
Figure 22: Transmission electron microscopy of a 21 day allograft coronary artery with large intercellular space within the media. Panel a. The media within the coronary artery contains large empty spaces where smooth muscle cells once occupied (stars). Asterisks indicate external elastic lamina. Numerous cell types were also present including monocytes, macrophages, as well as collagen. Scale bar = 10 μm. Panel b. Macrophage (star) within the media surrounded by intercellular space and smooth muscle cells. Scale bar = 2 μm. Panel c, d. Higher magnification images of macrophages with the presence of primary lysosomes. Scale bars = 2 μm.
Compared to previous time points, there was a dramatic increase in the presence of immune cells within the subendothelial space, the majority of which were monocytes and macrophages. Lymphocytes were also present within the media (Figure 23). These lymphocytes were characterized as round or club-shaped cells with a large, dark spherical nucleus, and sometimes with an indentation, densely compacted nuclear chromatin, and a scant cytoplasm. In addition, an elongated smooth muscle cell containing numerous vacuoles was also present within the media (Figure 23). Within the deep media, there were large areas of intercellular space between smooth muscle cells with occasional lymphocytes present (Figure 23). These large intercellular spaces were present between vacuole containing smooth muscle cells (Figure 24a,b). Fibroblasts were also observed within the media (Figure 24b, 25a,c). Collagen was much more conspicuous within the media at 21 days within allograft hearts (Figure 25a,c). A macrophage can also be seen adhering to a dying smooth muscle cell (Figure 25b) and therefore suggesting a reparative process beginning to form.
Figure 23: Transmission electron microscopy of a 21 day allograft coronary artery with the full length of an elongated smooth muscle cell. Note the presence of numerous vacuoles within the cell. There is also a dramatic increase in immune cell presence consisting primarily of monocytes and macrophages with occasional lymphocytes (arrow). Scale bar = 10 μm.
Figure 24: Transmission electron microscopy of a 21 day allograft coronary artery with increased intercellular space within the media. Panel a. Empty spaces where smooth muscle cells were once present (stars). Smooth muscle cells also contained vacuoles (arrows). Scale bar = 2 μm. Panel b. Fibroblast within the media neighbouring the internal elastic lamina (asterisk). Scale bar = 2 μm.
Figure 25: Transmission electron microscopy of a 21 day allograft coronary artery with collagen presence within the media. Panel a. Low magnification image of a coronary artery media with collagen presence (arrowheads) where smooth muscle cells once occupied. Scale bar = 2 μm. Panel b. Macrophage (star) phagocytosing a dying smooth muscle cell adjacent to collagen fibers. Scale bar = 2 μm. Panel c. Higher magnification image shows evidence of fibroblast presence within the media (asterisks) neighbouring collagen fibers (arrowheads). Scale bar = 2 μm.
Apoptotic cells were observed within the media both at early stages, characterized by nuclear chromatin condensation (Figure 26a, b), and late stages, characterized by apoptotic bodies and cytoplasmic fragmentation (Figure 26c). In addition, some cells within the media were undergoing mitosis, as indicated by the presence of the classic metaphase plates (Figure 26d). The presence of both apoptosis and mitosis at 21 days post-transplantation indicates high cell turnover and may also explain the remodeling of the media. Platelets, identified by the presence of α-granules, were observed adherent to the surface of disrupted endothelial cells (Figure 27).

In addition to coronary arteries, a 21 day allograft aorta was also obtained to examine and determine the identity of the giant cells observed by SEM, as well as determining the population of smaller inflammatory cells. The giant cells observed by SEM were confirmed to be large multinuclear macrophages (Figure 28a). In some instances, these giant cells had multiple cytoplasmic extensions projecting into spaces within elements of the elastic lamina (Figure 28a,b). As a result, portions of the internal elastic lamina were degraded, with the breakdown products of elastin present where giant cell cytoplasmic extensions interdigitate with the internal elastic lamina (Figure 28b). The majority of smaller leukocytes observed by SEM on both the luminal surface and within the intima of aorta were mononuclear cells and macrophages (Figure 28c). Moreover, numerous immune cells were also observed within the aorta and underneath an intact endothelium, (Figure 28c). Such alterations within the vessel wall would have been obscured from view by SEM.
Figure 26: Transmission electron microscopy of 21 day allograft coronary arteries with cells undergoing various stages of apoptosis and mitosis. Panels a,b. Cell within the media undergoing the early stages of apoptosis with nuclear chromatin initiating condensation (arrowheads). Scale bar = 2 μm. Panel c. Cell within the media undergoing a late stage of apoptosis, with the formation of an apoptotic body (arrowhead) and membrane fragmentation (arrows) and a neighbouring cell in which nuclear chromatin condensation has already occurred. Scale bar = 2 μm. Panel d. A cell undergoing mitosis as indicated by the presence of the classic metaphase plate (arrows). Scale bar = 2 μm.
Figure 27: Transmission electron microscopy of a 21 day allograft coronary artery of a platelet (arrows) with visible α granules (arrowhead), adhering to the surface of the endothelial cell and a migratory monocyte (star) below. Scale bar = 2 μm.
Figure 28: Transmission electron microscopy from an allograft aortic segment at 21 days post-transplantation. Panel a. *En face* section of a giant multinucleate macrophage (nuclei at arrowheads) and neighbouring elastic lamina (stars). Magnification 1400X. Panel b. Multiple cytoplasmic extensions of the giant cell (arrows, panels a and b) interdigitate with the elastic lamina (stars), fragmenting the elastin. Magnification 7700X. Panel c. Multiple mononuclear cells (arrowheads) were present beneath an intact endothelium (arrows). Magnification 1500X. Panel d. A large mitotic cell with metaphase plate (arrows) between elastic laminae (stars). Magnification 2840X.
As seen within coronary arteries at 21 days post-transplantation (Figure 26), some of the cells within the media of aorta were also observed undergoing mitosis, as indicated by the presence of the metaphase plates within rounded up cells (Figure 28d).

At 42 days post-transplantation, there were large regions of the coronary artery walls from which the endothelium was absent, resulting in the exposure of the internal elastic lamina (Figure 29a). Furthermore, there were regions within the internal elastic lamina that was severely damaged (Figure 29b) and disorganized (Figure 29c), resulting in exposure of medial smooth muscle cells to the vascular lumen (Figure 29b). This also resulted in unevenness of the luminal surface of the coronary arteries with exposed elastic lamina and smooth muscle cells. The intima also appears to be thickened, consisting mainly of immune cell infiltrates (Figure 30a). There was also a large population of inflammatory cells, predominantly monocytes and macrophages, present within the media (Figure 30b). Monocytes can also be seen both adherent to the endothelial cell surface (Figure 31a,b) and migrated into the intimal space (Figure 31b). Therefore, both the intima (Figure 31c) and media (Figure 31c) contained immune cell infiltrates. Several cells within the media and adventitia were also observed to be necrotic, with evidence of cell lysis, releasing their cellular contents within the interstitial space of the vessel wall (Figure 32). Finally, platelets were observed adherent to the endothelium, with the presence of dense platelet α-granules (Figure 33a,b) and features consistent with the open canalicular system (Figure 33b).
Figure 29: Transmission electron microscopy of 42 day allograft coronary arteries with large areas of denuded endothelium and disruptions to the internal elastic lamina. Panel a. Endothelial denudation results in exposure of the elastic lamina (arrows) and unevenness of the luminal surface. Scale bar = 10 μm. Panel b. Area where denuded endothelium resulted in damage and disruption to the internal elastic lamina (arrow) and exposure of medial smooth muscle cell (arrowhead) to the vascular lumen. Scale bar = 2 μm. Panel c. Higher power reveals a monocyte (star) adherent to an endothelial cell and the exposed disrupted internal elastic lamina (arrows). Scale bar = 2 μm.
Figure 30: Transmission electron microscopy of 42 day allograft coronary arteries with a thickened neointima and a large immune cell presence within the vessel. Panel a. The thickened intimal consists of immune cell infiltrates (stars). Scale bar = 2 μm. Panel b. Smooth muscle cells within the media appear to have lost their organized arrangement as a result of an immune cell presence consisting of monocytes and macrophages (stars) and a cell within the media undergoing necrosis (asterisk). Scale bar = 2 μm.
Figure 31: Transmission electron microscopy of 42 day allograft coronary arteries with monocytes both adherent to the endothelial cell surface and migrated within the intima. Panel a. High power image of a monocyte adherent to the endothelial cell surface. Scale bar = 500 nm. Panel b. Monocytes can be seen both adherent and migrated within the neointimal space (asterisk). Scale bar = 2 μm. Panel c. The intima (I) and media (M) have a large population of immune cell infiltrates consisting mostly of monocytes. Scale bar = 2 μm.
Figure 32: Transmission electron microscopy of 42 day allograft coronary arteries with cells within the deep media undergoing necrosis (asterisk), resulting in cell lysis and release of their cellular contents within the intercellular space of the coronary vasculature. Scale bar = 2 µm.
Figure 33: Transmission electron microscopy of 42 day allograft coronary arteries with platelets adherent to the endothelial cell surface and the presence of dense platelet granules (thin arrows) (Panels a and b) and features consistent of the open canalicular system (thick arrows) (Panel b). Scale bars = 500 nm.
3.3.3 Immune Cell Infiltration

In non-immunosuppressed allograft animals, immune cell infiltration was present as early as 1 day post-transplantation, as observed using TEM, with infiltrating monocytes migrating into the subendothelial space. At 4 days post-transplantation, a prominent leukocyte presence was observed and adherent to the endothelial cell surface by SEM. These leukocytes were identified as monocytes and macrophages using TEM and by 21 days post-transplantation, were seen underneath both intact and disrupted endothelium. As part of the immune response, giant cells were observed at 21 days post-transplantation exhibiting a ruffled cell morphology. Using TEM, these giant cells were further characterized as multinucleated macrophage with multiple cytoplasmic extensions. These cytoplasmic extensions could also be seen protruding towards and interdigitating with the internal elastic lamina. By 42 days post-transplantation, there was a multifarious immune cell presence with monocytes, macrophages, and lymphocytes present within the subendothelial space of both aorta and coronary arteries, the majority of which were monocytes and macrophages.
4 DISCUSSION

4.1 OVERVIEW OF STRUCTURAL FINDINGS

The nature of endothelial injury as one of the very early stages of transplant atherosclerosis in a Lewis-to-F344 rat heterotopic cardiac transplant model was examined in this study. Coronary arteries and aorta of non-operated control, allograft, and syngraft hearts were examined at the light microscopic level using en face silver nitrate staining, and at the ultrastructural level using SEM to assess endothelial integrity and injury and immune cell presence topographically, and by TEM for cross sectional and cellular ultrastructural analysis and disease progression. Silver nitrate staining is one of the oldest histological techniques, allowing one to visualize endothelial cell borders and surfaces with the distinctive cobblestone pattern of silver lines that the stain produces (128). In addition, any regions of endothelial denudation would also result in silver deposition and staining, as basement membranes in general are susceptible to staining with silver (110, 112, 127, 129, 130). Segments of aorta, right epicardial coronary, ventricular septal arteries, and left anterior descending coronary arteries from non-transplanted control, allograft, and syngraft hearts were studied. Within our control arteries, there was no apparent difference between saline, vehicle and cyclosporine treatment conditions. Therefore, it can be deduced that our treatment conditions did not have a negative effect on the coronary and aortic endothelium.

Our data suggest that substantial endothelial injury and damage may occur as early as 1 day post-transplantation in non-immunosuppressed
allografts, as observed at the light microscopic and ultrastructural levels. This was highlighted in our study with the presence of interendothelial gaps and areas of endothelial denudation. Early endothelial injury, with large regions of denuded endothelium and small residual patches of endothelial cells, has also been reported by others (110, 112) within rat aortic allografts but not within coronary arteries of rat heterotopic cardiac allografts.

The exact mechanism of endothelial gap formation and closure is still poorly understood. Endothelial gaps may occur as a result of contraction of adjacent endothelial cells involving actin and myosin filaments closely associated with the endothelial cell junction (131-133). Endothelial cytoplasmic extensions within the cell margin may be involved both during the formation and closure of endothelial gaps (134, 135). Morphological changes within the endothelium such as endothelial cell bleb formation, cell retraction, intercellular gap formation, cell detachment and denudation have been demonstrated to occur within minutes post-transplantation in rat lungs exposed to hypothermic and ischemic conditions in the absence of immunosuppressive therapy (136), indicating the rapidity of endothelial injury and the prophylactic effects of early immunosuppression. Within our non-immunosuppressed allografts, endothelial gap formation and basal lamina exposure resulting from endothelial cell death could also be a result of ischemia and reperfusion injury, compounded by the allogeneic immune response. Treatment of our allografts with cyclosporine ameliorated such injury. This may be partially attributed to the anti-apoptotic effects of CsA, inhibiting mitochondria transition pore formation (137), combined with it’s known
immunosuppressive properties. Importantly, pre-treatment with CsA can reduce I/R injury by decreasing the production of pro-apoptotic genes Fas, Fas-ligand, caspase 1 and 3 (138), as well as decreasing the expression of cytokine genes IL-1 and TNF-α (138) and inhibiting nuclear factor κB, a central transcription factor mediating inflammatory injury (139). Opening of the mitochondrial permeability transition pore (mPTP) during reperfusion causes mitochondrial swelling, leading to both apoptotic and necrotic cell death (140). CsA inhibits the opening of the mPTP, and therefore has a protective effect against reperfusion injury within the heart (141-143).

We also observed endothelial cell exfoliation by SEM and TEM, with detached endothelial cells both free within the lumen and adherent to the basal lamina. Endothelial cell fragmentation and detachment from the basement membrane are features consistent with necrotic endothelial injury (144). We also observed evidence of necrotic cell bursting and lysis within our non-immunosuppressed allografts, resulting in expulsion of their cellular contents into the interstitial space of the vessel wall. This can result in an enhanced and exacerbated inflammatory response with further recruitment of inflammatory cells leading to immune mediated cell death (145) and extensive tissue damage within the surrounding space (82). Severe tissue damage and necrosis as a result of the allograft response have been reported as early as 48 hours post-transplantation (146), whereas syngrafts had markedly reduced cellular injury and necrosis (146). Vacuoles within allograft endothelial cells were observed as early as 1 day post-transplantation. Cytosolic vacuolization, rupture of
endothelial plasma membranes, cell swelling and destruction of leaky outer membranes with poorly stained cytoplasm are all indicative of necrosis (144, 147, 148) and upon stimulation, can be detected as early as 8 hours ultrastructurally (147) indicating a rapid cellular response. We also observed apoptotic cells within the media at both early and late stages at 21 days post-transplantation. Apoptosis within coronary arteries is an important contributor to the pathogenesis of atherosclerosis and TVD (149). Early-stage apoptosis is characterized by homogeneous condensation of nuclear chromatin into sharply delineated granular masses with margination against the nuclear membranes and a well conserved cellular ultrastructure (147, 150). Late-stage apoptosis is characterized by nuclear chromatin condensation, nuclear fragmentation, shrinkage of the cell and condensation of the cytoplasm, detachment of the cell from the surrounding tissue, membrane blebbing, and apoptotic body formation (147, 150). Cells within allograft coronary arteries and aorta were also observed undergoing mitosis with the presence of the classic metaphase plate. Together with apoptosis, this suggests that our allografts were undergoing continual cellular turnover and may be indicative of a reparative response.

Platelet adhesion, activation, and spreading were observed by SEM within our allografts as early as 4 days post-transplantation on areas of apparent endothelial exfoliation with exposed extracellular matrix and elastic lamina. The subendothelial extracellular matrix is known to be thrombogenic and to initiate recruitment of circulating platelets to the injured vessel wall (151). However at later time points, we observed platelets adherent to both exposed extracellular
matrix and to allograft endothelium. This may have occurred as platelets can adhere to dysfunctional endothelium (152). These activated platelets may then release their granules containing pro-inflammatory compounds and induce inflammatory reactions (151), or release growth factors such as platelet-derived growth factor (PDGF) which may contribute to the migration and proliferation of smooth muscle cells and monocytes and their synthesis of extracellular matrix components (153) and thereby abet and exacerbate the development of TVD. Activated platelets in the blood can bind to leukocytes, with a preference to monocytes, resulting in the formation of platelet-leukocyte aggregates (154). Within the allograft milieu, platelet-leukocyte aggregation may play a larger role in the development of TVD with the high prevalence of leukocytes within the blood stream, adherent to the endothelium. Moreover, it has been suggested that increased platelet aggregation is a predictor of acute coronary events among heart transplant patients (155).

4.2 ENDOTHELIUM

Alterations of Endothelial Integrity

To our knowledge, our study is the first to document ultrastructural characteristics of endothelial injury and activation in coronary arteries within heterotopically transplanted rat hearts. We observed alterations in endothelial integrity as early as 1 day post-transplantation in non-immunosuppressed allografts. Utilizing both scanning and transmission electron microscopy, ultrastructural changes in endothelial topography and integrity were observed in
saline-treated rat allograft aortas and coronary arteries as early as 1 and 4 days post-transplantation. Cytoplasmic extensions of varying lengths were detected both by SEM and TEM on the luminal endothelial cell surface. The production of such irregular plasma membrane extensions is consistent with other descriptions of endothelial injury and activation (156, 157) as well as during gap formation (134). Some of these cytoplasmic extensions overlapped endothelial cell junctions. These cytoplasmic extensions may form after leukocyte transmigration (157) and may also be indicative of the attempt to close interendothelial gaps (134, 157). We also observed allograft endothelial cells with a roughened luminal plasma membrane which can occur as a result of cytokine exposure (158). Such irregular adluminal plasma membrane protrusions are features consistent with endothelial activation (156, 158).

We detected an abundance of rough endoplasmic reticulum and mitochondria within endothelial cells as early as 1 day post-transplantation using TEM suggesting cellular activity and metabolism (156). Endothelial activation, resulting in an increase in cytoplasmic organelles and vesicle appearance, has been detected as early as 6 hours after endothelial injury (156) indicating a rapid endothelial response to injury. In our study, we detected numerous caveolae on the luminal surface of endothelial cells at 1 day post-transplantation, consistent with features of increased cellular activity (156). Similar to our findings, increases in endothelial irregularities with luminal cytoplasmic extensions accompanied with an increase in caveolae and cytoplasmic organelles, endothelial vacuolization, interendothelial gaps, along with cell loss have been
reported 1 to 2 days post-endothelial injury using an atriovenus fistula to induce endothelial injury and activation (156).

We also observed as early as 1 day post-transplantation, monocytes and neutrophils transmigrated within the subendothelial space. The presence of inflammatory cells may participate in the phagocytosis of necrotic debris, and monocyte and macrophage margination, which can lead to changes in endothelial integrity. Treatment with CsA both in vitro and in vivo can decrease adhesion molecule expression within endothelial cells (84, 159, 160) and attenuate early leukocyte adhesion and accumulation (84) which may abate monocyte mediated injury. This may partially explain the protective effects of CsA early post-transplantation within our allograft hearts.

4.2.2 Implications for Endothelial Permeability and Uptake of Circulating Molecules

We have observed intercellular disruptions of endothelial integrity that could result in an increase in interendothelial permeability. As a result of the inflammatory response, vascular permeability can increase and the leakage of fluid and macromolecules from the blood can readily enter the interstitial space and give rise to tissue edema associated with inflammation.

Inflammatory mediators such as cytokines can rapidly induce the formation of gaps between endothelial cells (103, 131) and have been shown to increase the permeability of endothelial monolayers (161-163). In addition, we have found evidence that intercellular gap formation may begin at tricellular
corners within interendothelial junctions, originally predicted by Walker et al (164). Together, such endothelial disruptions may facilitate entry of macromolecules such as LDL and possibly VLDL, as well as inflammatory cells, plasma proteins, and fluid into the subendothelial space. Within human cases of TVD, prominent LDL and VLDL accumulation has been documented (95, 165) along with associated apolipoproteins E, (a), and B (166). Once within the subendothelial space, such an environment fosters their entrapment within the extracellular matrix, notably to proteoglycans such as versican (166-168). In native atherosclerosis development, the predominant lipoprotein particles are LDL - particularly small and dense LDL particles (169); VLDL particles may play a lesser direct role due to their larger size (170). In considering the great endothelial disruption, increased vascular permeability in the allograft setting may allow larger numbers of lipoprotein particles (both LDL and VLDL) to enter the sub-endothelial space with a higher influx rate (170). Oxidative modification of these entrapped lipoproteins may occur, resulting in their uptake by macrophages through scavenger receptors (165, 171, 172). These stages can be further accelerated post-transplantation due to the increased oxidative stress and dyslipidemia associated with transplantation (173-175). The altered integrity to the endothelium and permeability can then lead to further infiltration of macrophages, T lymphocytes and other molecules within allografts and thereby accelerate the initiation and progression of TVD.
4.3 INFLAMMATORY CELLS

4.3.1 Inflammatory Cells Involved in TVD

We have observed monocytes undergoing diapedesis as early as 1 day post-transplantation suggesting that the immune response can act as one of the earliest host responses to the newly grafted organ. Gohra et al have also detected the presence of leukocytes attached to residual endothelial cells at 1 day post-transplantation by SEM in a rat aortic allograft model (112). In our model, CsA treatment apparently attenuated leukocyte adhesion and transmigration, indicating that an allograft response may be partially responsible for early endothelial injury and immune cell infiltration. In addition, lesions consisting predominately of mononuclear cells and some T lymphocytes have been observed as early as 3 days post-transplantation by SEM within an aortic allograft model (176), further supporting the significance of an inflammatory response as an early step in the initiation of TVD. Due to their interface with the blood, endothelial cells within allografts are early targets of immune-mediated injury. Recently, it has been shown in murine cardiac allografts that a T-cell mediated attack on the allograft endothelium can occur within hours following organ reperfusion, amplifying inflammation and necrosis (146). There was also a striking increase in polymorphonuclear leukocytes (PMN) infiltration within these allografts, whereas inflammation resolved quickly within syngrafts (146), suggesting the ability of the recipient to detect and differentiate the presence of allogeneic tissue as early as 12 hours post-transplantation. Tissue damage and necrosis were also markedly more apparent within allografts as early as 48 hours.
post-transplant within their study (146). Within our allografts, we also observed both monocytes and neutrophils within the subendothelial space as early as 1 day post-transplantation. Together, this illustrates the importance of the allogeneic response as an early if not first stage in the initiation of TVD.

Recently, it has been reported that an early CD8$^+$ T cell-mediated immune response to cardiac allografts can occur before detectable alloreactive T cell priming (146). It has also been suggested that only CD8$^+$ T cells, and not CD4$^+$ T cells, likely play a role as resting murine endothelium lack MHC class II expression (146). However, CD4$^+$ T cells may have an additional compounding effect, as humans constitutively express detectable levels of MHC class II within the vascular endothelium including coronary arteries (88, 177). Moreover, MHC class II expression is upregulated in both human and rat allograft endothelium and was associated with early adherence and subendothelial accumulation of monocytes and T cells (10, 12, 77). It has also been suggested that this early CD8$^+$ T cell response is unresponsive to cyclosporine (146). An alternative explanation may be the murine model's unresponsiveness to cyclosporine therapy (114, 115). Taken together, our rat allograft model may have undergone an aggressive immune attack within hours following reperfusion and ensuing early inflammation resulting in an accelerated immune response.

In our study, large populations of monocytes and macrophages were observed within the subendothelial space within both an intact and disrupted endothelium at 21 days post-transplantation and further progressing by 42 days post-transplantation within non-immunosuppressed allograft hearts. Monocytes
and macrophages play an important role in the pathogenesis of TVD with release of their cytokines contributing to lesion development. Lymphocytes were also present within our allografts, albeit in lower numbers. Previous studies have shown a large monocyte and macrophage presence along with T lymphocytes from 7 to 20 days post-transplantation within rat aortic allografts, accumulating both on and underneath the endothelium and forming an intimal lesion (112). Our study indicates that this response may occur even earlier using ultrastructural methods of detection. Early endothelial expression of MHC class II and ICAM-1 as a result of the allograft response is also associated with the early adherence and subendothelial accumulation of monocytes and T lymphocytes (10). Once within the subendothelial space, macrophages and T lymphocytes can directly injure endothelial cells, release cytokines and promote an ongoing inflammatory response to the expanding neointima, advance atherogenesis, and facilitate TVD. Within transplant patients, these features may be accelerated with an even larger presence of T lymphocytes and macrophages from the allogeneic immune response.
4.4 DISEASE PROGRESSION

It has been suggested that early mononuclear cell accumulation in the vessel wall triggered by a minor histocompatibility mismatch, such as those of the Lewis-to-F344 model, leads to mesenchymal cellular proliferation and collagen deposition, resulting in diffuse fibro-obliterative coronary lesions (113). These events can contribute to intimal thickening, a prominent feature of TVD (95, 110, 112). Within the Lewis-to-F344 cardiac allograft model, a distinct inflammatory stage precedes smooth muscle cell accumulation in areas of intimal thickening, suggesting that mononuclear cells play a key role in the developing lesion (10). Our observations of early monocyte diapedesis may be one of the initial contributors resulting in intimal thickening, disease progression, and consequently, TVD. In parallel with our study, two-week-old grafts of Lewis-to-F344 cardiac allografts consist of diffuse, dense interstitial mononuclear cell infiltrates, which are composed predominantly of macrophages (>75%) and T cells (10-20%) with interleukin-2 (IL-2) receptor positivity (10, 178). By 42 to 70 days post-transplantation, grafts exhibited persistent and severe mononuclear interstitial cell infiltrates and wide areas of infarction (10). A similar intense inflammatory response has been detected within 2 weeks post-transplantation in rat (111, 112, 176) and rabbit non-immunosuppressed aortic allografts (179). The majority of allograft studies use immunohistochemical methods of immune cell identification. Our study augments such work with ultrastructural detection of immune cell infiltrates and disease progression. This allows for a more sensitive method to detect the initial endothelial injury and identify the low levels of
immune cell presence that may occur early post-transplantation that would
normally evade light microscopic immunohistochemical detection. Subsequently,
our study within cardiac allograft aorta and coronary arteries indicates that the
inflammatory response may occur even earlier post-transplantation than
previously reported and may play a larger role in early disease initiation and TVD
development. Clinical studies have shown that acute rejection episodes are a
strong predisposing risk factor for the development of TVD (146). Findings within
the Lewis-to-F344 model are consistent with late findings in human allografts and
document an early mononuclear inflammatory stage preceding intimal smooth
muscle cell accumulation in chronically rejecting Lewis grafts (10). This supports
the hypothesis that mononuclear cell release of cytokines may promote an
ongoing inflammatory response to the expanding intima and smooth muscle
proliferation in graft arteriosclerotic lesions (7, 12, 37).

Moreover, a single dose of CsA to both the donor and recipient has been
shown to inhibit left ventricular dysfunction as a result of prolonged I/R (180).
CsA may also reduce the effectiveness of monocytes, macrophages and T
lymphocytes, and the immunosuppressant may have direct effects of attenuating
the allograft immune response and early endothelial injury (69, 86). Within our
study, either cessation of CsA therapy or lack of CsA (saline and vehicle)
treatment was associated with the appearance of endothelial abnormalities (36).
Elimination of CsA from plasma may take several days in rats (126, 181),
therefore, our observations of CsA-treated rats at 21 days post-transplantation,
and thus a net 7 days after cessation of cyclosporine treatment, may
underestimate the potential for injury in the absence of the drug. Discontinuation of cyclosporine therapy was associated with significant endothelial damage, the presence of leukocytes on and below the endothelium, and activated platelets adherent to exposed extracellular matrix (36). This was particularly pronounced with our SEM data where we observed leukocytes infiltrating the subendothelial space underneath an intact endothelium in our 21 day cyclosporine-treated allografts (36). These results are supported by other work where cessation of cyclosporine therapy after 20 days of treatment (12mg/kg per day) was associated with a significant increase in mean vessel occlusion (up to 70%) in the Lewis-to-F344 cardiac transplant model (126).

As we demonstrated ultrastructurally in allograft coronary arteries, others have reported progressive intimal thickening consisting of a large number of $\alpha_1$-actin, desmin and vimentin-positive myofibroblastic cells at 21 days post-transplantation in rat aortic allografts (176). Other studies have reported intimal thickening in rat aortic allografts as early as 14 days post-transplantation (111, 112). We observed smooth muscle cells within the media with highly organized, close cell-to-cell interactions within non-operated controls and early post-transplant within non-immunosuppressed allograft coronary arteries. However, by 21 days post-transplantation, there was a trend towards an increase in interstitial space between smooth muscle cells, the presence of fibroblasts within the media and extracellular matrix components of collagen fibrils and elastic fibers. Similarly, it has been previously shown by TEM that the intima of 14 day aortic allografts contained scant amounts of extracellular matrix and the media
contained SMC-like cytoarchitecture with collagen fibrils surrounding them (182). In our study, we also detected fibroblasts within the media and collagen was much more prominent at 21 days post-transplantation within coronary arteries of allograft hearts. Activated fibroblasts of recipient origin have been detected within Lewis to F344 cardiac allografts (183). Although fibroblasts play an important role in wound healing, their exact role in allograft fibrosis and chronic rejection is still not completely understood (183). Our features of fibroblast and extracellular matrix presence with collagen and elastic fibers surrounding smooth muscle cells at progressive time points post-transplantation are similar to characteristics observed after balloon injury (184) and may indicate a reparative response to the allograft condition. Furthermore, cyclosporine treatment has been shown to reduce fibroblast proliferation and therefore, may have a further protective effect within allografts (185). This attribute of excessive deposition of extracellular matrix components within the growing neointima is a prominent feature of TVD in both animal models and in humans (167, 186).

4.5 SUMMARY OF SIGNIFICANT FINDINGS

To our knowledge, this study is the first to document, in extensive detail, the ultrastructural characteristics of early endothelial injury post-transplantation within allograft coronary arteries and aorta. Substantial early endothelial injury was observed within non-immunosuppressed allografts in the form of interendothelial gaps and large areas of endothelial denudation with exposure of basal lamina. Platelets were adherent to both regions of denuded endothelium
and to the endothelial surface, and were reflective of altered endothelial integrity. We also observed holes at tricellular corners within allograft endothelium, a possible site of increased transendothelial permeability together with interendothelial gaps. Prophylactic treatment of allografts with CsA reduced endothelial injury, gap formation and endothelial denudation.

Endothelial cell vacuolization was observed early post-transplantation, whereas smooth muscle cell vacuolization occurred at 21 days post-transplantation. Ultrastructural evidence of endothelial activation with increased cellular activity due to the allograft response was observed as early as 1 day post-transplantation with the presence of RER, mitochondria and caveolae. Cytoplasmic extensions of endothelial cell borders were also present. Smooth muscle cell disorganization with an increase in interstitial space within the media was also observed within allograft coronary arteries. Evidence of cellular death with the presence of cells undergoing apoptosis and necrosis, as well as cells undergoing mitosis, were observed by 21 days post-transplantation within allograft aorta and coronary arteries. Fibroblasts and collagen were also observed within allograft arteries and together with apoptosis and mitosis.

The presence of monocytes and neutrophils transmigrated within the subendothelial space was observed as early as 1 day post-transplantation in non-immunosuppressed allograft coronary arteries. We observed neointima formation and disease progression in both aorta and coronary arteries consisting predominately of monocytes and macrophages and some lymphocytes by 21 days post-transplantation. Giant cells with cytoplasmic extensions interdigitating,
fragmenting and degrading the elastic lamina were also observed. In our study, CsA treatment attenuated leukocyte adhesion and transmigration along with endothelial damage.
CONCLUSIONS

We have shown that loss of endothelial integrity through endothelial cell death and intercellular gap formation accompanied by the infiltration of leukocytes are among the earliest responses in the host of newly grafted organs. In clinical settings where investigations of TVD are undertaken, there is often difficulty in obtaining tissues early post-transplantation, limiting studies investigating the role of early endothelial injury and disease initiation. Therefore, animal studies of TVD as our Lewis-to-F344 heterotopic transplant model allow the investigator to utilize a controlled experimental design to examine in depth early disease initiation and development. Using ultrastructural methods of detection, we provide evidence that the allograft damage may occur even earlier than previously reported. Our observations indicate the rapid development of TVD within allografts in which endothelial-based mechanisms may at least in part explain the phenomena involved. Early administration of cyclosporine ameliorates endothelial injury and prolongs graft survival. Our data also indicates that cyclosporine or similar immunosuppressive agents should be continued and optimized to achieve longer beneficial effects on endothelial integrity. Although the results of our study support the immune response as one of the earliest insults to allograft vessels, other mechanisms in both acute and chronic rejection should also be considered. Finally, our observations suggest the need for the continued regeneration of the injured endothelium within allografts. Whether they are derived from donor or host still remains to be elucidated. Methods of regeneration of the endothelium will be of great
therapeutic importance for prolonged allograft survival and other procedures resulting in endothelial injury such as balloon angioplasty with coronary stenting. Long-term maintenance of graft patency is particularly important for transplant patients and is critical as organ waitlists become longer, compounded by the short supply of suitable donors.
6 REFERENCES


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prevents the development of cardiac allograft vasculopathy. Transplantation 2002;73(7):1116-22.


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APPENDIX: LIST OF PUBLICATIONS, ABSTRACTS, ORAL PRESENTATIONS, AND AWARDS

REFEREED PAPERS


ABSTRACTS


Infection of Mouse Hearts. Presented at Molecular Mechanisms of Growth, Death, and Regeneration in the Myocardium: Basic Biology and Insights into Ischemic Heart Disease and Heart Failure (AHA), Snowbird, Utah, August 2003.


INVITED PRESENTATIONS


AWARDS

Best Overall Poster Presentation, Department of Pathology and Laboratory Medicine Annual Research Gala, The University of British Columbia, Vancouver, BC, May 2003.