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DEVELOPMENT OF INTIMAL HYPERPLASIA IN TRANSPLANT ARTERIOSCLEROSIS

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ABSTRACT

Vascular disease is the main cause of disability and mortality in the western world and the major limiting factor for long-term survival of transplanted organs. Occlusive vascular lesions lead to ischemia and structural changes in organs and in transplant arteriosclerosis and restenosis after endovascular procedures, narrowing of the vessel lumen is partly due to intimal hyperplasia caused by smooth muscle cells (SMCs). In this thesis, I have examined the mechanisms involved in intimal hyperplasia in a rat aortic allograft model of transplant arterioscleroris with an emphasis on the biology of SMCs in this process.

First, the phenotypic properties of the SMCs involved in the formation of intimal hyperplasia were studied by electron microscopy and immunohistochemistry. A segment of the abdominal aorta was transplanted orthotopically from Fischer to Lewis rats and the transplanted vessels examined after 1-12 weeks. After 1 week, loss of endothelial cells, adhesion of platelets and leukocytes to the luminal surface, and a phenotypic modification of SMCs in the media were observed. Subsequently, modified SMCs appeared in the intima and lymphocytes and macrophages were found to infiltrate the intima as well as the adventitia. This occurred together with detachment of endothelial cells and activation of SMCs in the media as determined by the induction of cellular retinol-binding protein-1. Later, SMα-actin positive SMCs were observed to migrate into the intima and proliferate as judged by staining for cyclin D1 and proliferating cell nuclear antigen. TUNEL- and Fas/CD95-positive SMCs, indicating apoptosis, appeared in the media which was followed by a reduction in SMα-actins taining in this layer. The continued development of the neointima was associated with a decrease in SMα-actin-positive SMCs, an increased staining for the extracellular matrix components fibronectin and osteopontin, and a further accumulation of inflammatory cells. The maximum growth in size of the neointima with an increase both in the number of SMCs and the content of extracellular matrix occurred 4-8 weeks after transplantation. SMCs and monocytes/macrophages in the neointima and in the media were also noted to accumulate lipid, turn into foam cells, and eventually show signs of necrosis and apoptosis. Within the lipid-rich cell remnants, calcification also occurred. Finally, after 12 weeks, the growth in mass of the intimal lesions ceased and reformation of an endothelial lining was detected.

In order to examine if SMCs derived from the host animal can participate in intimal hyperplasia, we transplanted aorta of F344 female rats to Lewis male rats with or without cyclosporin A treatment. As a control, one group of animals was transplanted with aortic isografts exposed to prolonged cold ischemia. Infiltration of SMCs and inflammatory cells into the intimal lesions, cell proliferation, and apotosis were analyzed by immunostaining and laser microdissection followed by real-time PCR for the SRY gene to determine cell origin. Early after transplantation, proliferating and apoptotic graft SMCs were observed in the neointima and leukocytes and immunoglobulins appeared in the grafts. At this time apoptosis of medial SMCs occurred and proliferating, SRY-positive, host-derived SMCs started to accumulate in the neointima. After 8 weeks, the neointima was mainly composed of host-derived SMCs. Immunosupression with cyclosporin A significantly decreased the number of host SMCs in the neointima and only a small number of host SMCs were observed in isografts exposed to prolonged ischemia. To explore the possible bone marrow origin of allograft cells, female LEW rats were irradiated and substituted with bone marrow from male LEW rats by transplantation of vascularized bone marrow or by infusion of bone marrow cells, followed by transplantation of aorta from female F344 rats. Immunostaining for cell-specific markers and real-time PCR for the SRY gene showed that the number of leukocytes was lower than the number of bone marrow-derived cells in intimal lesions. Primed in situ labeling for the SRY gene combined with immunostaining confirmed the presence of SM-like cells of male origin in the vessel wall in the intima. Similar observations were made after balloon injury of the carotid artery.

The findings add further support for the prior assumption that early loss of endothelial cells contribute to the initial response and activation of SMCs, whereas the inflammatory process may be the dominating factor that influence vessel structure later after transplantation. The observations also suggest that progenitors of bone marrow origin give rise to cells with SM-like properties during the formation of intimal hyperplasia after allotransplantation as well as after balloon injury. In addition, the results provide the basis for a novel theory of transplant arteriosclerosis which suggests that the development of intimal hyperplasia in this vasculopathy is a dynamic two-stage process that involves apoptosis of resident graft SMCs triggered by an allogenic immune response which also promotes recruitment of host-derived SMCs.

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To my family

LIST OF PUBLICATIONS

The thesis is based on the following original articles, referred to in the text by their Roman numerals

I. Bojakowski, K., Religa, P., Bojakowska, M., Hedin, U., Gaciong, Z., and Thyberg, J. 2000.
Arteriosclerosis in Rat Aortic Allografts: Early Changes in Endothelial Integrity and Smooth Muscle Phenotype
Transplantation 70:65-72

II. Religa, P., Bojakowski, K., Gaciong, Z., Thyberg, J., and Hedin, U. 2002.
Arteriosclerosis in Rat Aortic Allografts: Dynamics of Cell Growth, Apoptosis and Expression of Extracellular Matrix Proteins
Molecular and Cellular Biochemistry, in press.

III. Religa, P., Bojakowski, K., Maksymowicz, M., Bojakowska, M., Sirsjö, A., Gaciong, Z., Olszewski, W., Hedin, U., and Thyberg, J. 2002.
Smooth Muscle Progenitor Cells of Bone Marrow Origin Contribute to the Development of Neointimal Thickenings in Rat Aortic Allografts and Injured Rat Carotid Arteries
Transplantation 74, 1310-1315

IV. Religa, P., Bojakowski, K., Gaciong, Z., Thyberg, J., and Hedin, U. 2002.
Intimal Hyperplasia in Rat Aortic Allografts: Role of Allogenic Immune Response in the Recruitment of Host Derived Smooth Muscle Cells
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LIST OF ABBREVIATIONS

CMV	cytomegalovirus
CRBP-1	cellular retinol-binding protein-1
FGF	fibroblast growth factor
FISH	fluorescent in situ hybridization
HLA	human leukocyte antigen
ICAM	intercellular adhesion molecule
IFN	interferon
IGF	insulin-like growth factor
IL	interleukin
LCM	laser capture microdissection
LDL	low-density lipoprotein
LSCM	laser scanning confocal microscopy
MCP-1	macrophage chemoattractant protein-1
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
PRINS	primed in situ labelling
PTCA	percutaneous transluminal coronary angioplasty
RT PCR	real-time polymerase chain reaction
SM	smooth muscle
SMC	smooth muscle cell
SRY	sex related gene on chromosome Y
TGF	transforming growth factor
TNF	tumor necrosis factor
TUNEL	Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling
VCAM	vascular cell adhesion molecule
vWf	von Willebrand factor

INTRODUCTION

Replacement-organ transplantation is an established treatment for end-stage disease of the kidneys, heart, liver or lungs. With respect to kidney transplantation, which is the most common solid-organ transplantation, the procedure gives improved quality of life and higher survival rates for patients in comparison with dialysis. About 200 000 transplants from cadaver- and living donors have been performed every year in the USA since the first successful kidney transplantation in 1954. The 1-year survival rate of renal grafts is about 80-90% and the half-life is about 10 years (Cecka, 2000). The first cardiac transplantation was done by Dr. Christian Barnard in 1967 and has since become the treatment of choice for patients with severe cardiac dysfunction and life expectancy of less than 6-12 months. After cardiac transplantation, the survival rate is up to 85% after 1 year and 66% after 5 years (Keck et al., 1997).

Transplanted organs evoke an immune response with both cellular and humoral components, directed against tissue antigens incompatible with those of the recipient (Hancock et al., 2002; Mauiyyedi and Colvin, 2002). Therefore, recognition and rejection of the allograft by the immune system of the recipient has to be prevented by immunosuppressive drugs. The progressive development of immunosuppressive therapy after transplantation, for example through the introduction of azathioprine and prednisone in the sixties, cyclosporin in the eighties, and sirolimus and mycophenolate mofetil in the nineties has constantly improved the survival of grafts and patients, mostly with respect to short-term results (Gonin, 2000; Olyaei et al., 2001). However, the long-term survival after transplantation is still limited and a progressive deterioration of graft function occurs in 3-5% of all transplanted patients per year (Cecka, 2000; Keck et al., 1997; Wilkinson, 2001).

Chronic transplant dysfunction is the major limiting factor for successful outcome after transplantation of solid organs (Womer et al., 2000). The process involves occlusive arteriopathy known as transplant arteriosclerosis and atrophy with fibrosis of the organ parenchyma (Furness, 2001; Tejani and Emmett, 2001). Transplant arteriosclerosis is, in general terms, a reparatory remodeling of vessels in transplanted solid organs (also referred to as transplant vasculopathy). The process is characterized by infiltration of inflammatory cells in the vessel wall, a progressive narrowing of the vessel lumen due to a healing reaction in the intima, intimal hyperplasia, and deterioration of the media with cellular death and fibrosis (Furness, 2001).

The development of transplant arteriosclerosis has many features in common with other vascular diseases such as atherosclerosis and luminal narrowing after vascular interventions (Newby, 2000; Pasterkamp et al., 2000). Atherosclerosis is the most common cause of arteriosclerosis and cardiovascular diseases. Also this disease process can be regarded as a reparatory reaction to a vascular injury and begins with the formation of lipid streaks and inflammation in the artery wall. It then involves further lipid deposition and thickening of the inner layer of the arteries, the tunica intima. This gives rise to a lesion, an atherosclerotic plaque, that projects into the vessel lumen, and eventually restricts the flow of blood, leading to end-organ ischemia, for example in the heart (Ross, 1999). Cardiovascular diseases due to atherosclerosis are the main cause of death and disability in economically developed countries, even though the situation has improved since the seventies due to changes in living habits and development of new medical treatments (Oberman et al., 1994). However, it was recently concluded that a predicted increased survival of the general population will lead to increased morbidity and mortality because of cardiovascular disease and generate a rise in medical costs as well as a decrease in the quality of life (Amouyel, 2002; Mukherjee et al., 2002). The main risk factors for atherosclerosis are age, high blood pressure, smoking, high concentration of cholesterol and triglycerides in the blood, diabetes, obesity, and low physical activity. The morbidity is higher among men as well as some ethnic groups. Furthermore, it can be affected by drugs, narcotics, alcohol and hormonal replacement therapy (Sharabi et al., 2001). There is no established curative therapy for atherosclerosis available, although treatment of heart and extremity ischemia includes many pharmacological and surgical approaches. Albeit these efforts do not cure the underlying atherosclerotic disease, they have been shown to be effective in secondary prevention and in relief of the ischemic symptoms, thereby significantly altering morbidity and mortality in cardiovascular disease (Dawson et al., 2002). Vascular interventions, such as bypass surgery and percutaneous transluminal coronary angioplasty (PTCA), with or without stents, are the most common treatments of myocardial ischemia today (Mulukutla and Cohen, 2002; Yutani et al., 1999). The number of vascular interventions by intravascular catheterisation is increasing and has recently reached about 1 million interventions per year worldwide (Bittl, 1996). Even if the early outcome is very promising, about 30-50% of patients after PTCA and 10-15% after bypass surgery need a renewed surgical treatment within one year because of a recurrent narrowing due to intimal hyperplasia and vessel remodeling, also referred to as restenosis and graft stenosis (Sallam et al., 2001).

Intimal lesions lead to clinically important vascular narrowing in transplant arteriosclerosis and after surgical procedures. However, in some cases an enlargement of the vessel may occur, i.e. a compensatory positive remodeling (Moliterno and Topol, 1998). Formation of intimal hyperplasia is also involved in the formation of a fibrous cap in atherosclerosis which provides stability to the lesions (Libby, 2000). Since the formation of the intimal hyperplasia is a common phenomenon in vascular pathology, the understanding of this process will be crucial for the future development of clinically useful treatment strategies in the management of a large variety of vascular diseases (Galis et al., 2002). In this thesis I have provided novel information related to the mechanisms of intimal hyperplasia in a rat aortic transplant model. This information will hopefully be important for the future development of new therapeutic strategies.

NORMAL ARTERIES

Normal arteries have a three-layer structure with an intima, a media and an adventitia. The intima is the most internal layer and is covered by a sheet of endothelial cells lining the vessel lumen and forming a barrier against the blood. The endothelial cells form a tight monolayer and rest on a basement membrane, a fine meshwork made up of extracellular matrix components, mainly collagen type IV, laminin, and heparan sulfate proteoglycans (Gutierrez et al., 1998; Thyberg, 1998). Occasional SMCs may be present in the intimal space underneath the endothelium. The media is composed of circular layers of SMCs alternating with bundles of elastin arranged in concentric lamellae. The number of layers is dependent on the size of the vessel. The sheets of elastin are fenestrated and associated with loosely packed collagen fibrils. SMCs are the major cellular components of the arterial media, although some elastic and muscular arteries also contain vasa vasorum, i.e. capillaries which penetrate from the adventitia and provide nutrients and oxygen. The SMCs are responsible for synthesis of elastin, collagen and other extracellular matrix components. These muscle-like cells are influenced by factors released from the endothelium, nerves, circulating blood, and the surrounding extracellular matrix. They are electrically connected to various extents by gap junctions. The external part of the arterial wall is formed by the adventitia, a thin connective tissue mainly composed of fibroblasts and a collagen-rich extracellular matrix. The adventitia contains vasa vasorum, a lymphatic system, and some nerve fibers involved in vasomotor control (Ross et al., 1995).

RESTENOSIS

Development of restenosis is a complex process that leads to vascular narrowing after surgical procedures on vessels. It occurs after atherectomy, balloon angioplasty and stenting (Kataoka et al., 2002). A related process also takes place in by-pass grafts of autologous veins. Restenosis is the major cause of long-term failure after vasular intervention. Up to one half of the patients need repetitive procedures due to recurring narrowing after coronary balloon angioplasty (Sallam et al., 2001). Whereas restenosis is a process which develops in diseased arteries, stenosis in by-pass grafts of autologous veins take place in a previously healthy vessel and accounts for the major cause of long-term graft failure after by-pass surgery (Waller et al., 1984). The process is initiated by the mechanical and hemodynamic injuries which the surgical procedure imposes on the vessel segment (Depre et al., 1998), and in simple terms, restenosis can be divided into two separate events. The first is a vascular healing and involves early platelet adhesion, SMC activation and migration from the media to the intima, where subsequent cell proliferation and deposition of extracellular matrix form a lesion or thickening, i.e. intimal hyperplasia (Pasterkamp et al., 2000). The second part of the process involves remodeling of the vessel wall as an adaption to altered shear forces, tissue scarring and fibrosis (Pasterkamp et al., 2000). Clinically, both pharmacological and surgical techniques have been introduced to prevent narrowing after vascular intervention. Antiplatelet and antithrombotic strategies have improved the early outcome and the introduction of stents has increased patency after PTCA by limiting negative remodeling (Schwartz and Seidelin, 1995; Virmani et al., 2002). However, the problem of intimal hyperplasia after vascular reconstruction has remained unsolved and trials using systemic treatment with either calcium channel blockers, angiotensin converting enzyme inhibitors, lipid lowering drugs, antioxidants, or immunosuppression by cyclosporin have been unsuccessful (el-Sanadiki et al., 1990; Landymore et al., 1990; Landymore et al., 1989; Mohacsi et al., 1997; Okimoto et al., 2001). Recently, the introduction of drug-eluting stents after coronary angioplasty has emerged as a promising new treatment strategy and local delivery of sirolimus has been shown to reduce in-stent restenosis rates after coronary revascularisation with high efficiency (Regar et al., 2002).

TRANSPLANT ARTERIOSCLEROSIS AND THE MECHANISMS OF TRANSPLANT REJECTION

The pathology in allografts with chronic dysfunction is found in the vascular bed and in the parenchyma of the organ (Kouwenhoven et al., 2000). For example, intimal hyperplasia in vessels and fibrosis of the parenchyma is observed in transplanted hearts and may lead to congestive heart failure, arrhythmias, myocardial infarction, and sudden

death (Rebocho et al., 2001). In renal allografts, tubular atrophy with glomerulosclerosis and interstitial fibrosis may lead to a progressive loss of renal function (Paul, 1995). The knowledge about the pathogenesis of allograft vascular disease and transplant arteriosclerosis has been obtained from animal experiments such as the rat aortic transplantation model which was introduced in the sixties and is still widely used (Rossmann and Lacha, 1999; Urist and Adams, 1967). In comparison to solid allografts, this model allows studies of vascular disease alone after transplantation with a limited influence from the parenchyma, although inflammation in the adventitia is a common finding (Rossmann et al., 1999). Transplant arteriosclerosis is usually described as an occlusive vascular disorder with stenosis due to intimal hyperplasia and medial necrosis (Pethig et al., 2000). However, luminal loss in allograft vessels can develop without any signs of medial necrosis and even without changes in endothelial integrity (Liu and Butany, 1992). Furthermore, studies with intravascular ultrasound have indicated that luminal loss can also be caused by inflammation and fibrosis in the adventitia and negative remodeling of the vessel (Lim et al., 1997; Schwarzacher et al., 2000). The immunological reaction involves a sequential cellular infiltration by leukocytes, mostly macrophages and lymphocytes (Plissonnier et al., 1995), the appearance of immunoglobulins, and expression of major histocompatibility complex (MHC; (Wood et al., 1988). In the rat model, complete remodeling with intimal hyperplasia, medial destruction, and a complete loss of any physiological function is observed after 8-12 weeks (Bigaud et al., 1999). The development of transplant arteriosclerosis is presented in Fig. 1. Geraghty and co-workers described phenotypic modulation of SMCs and indirectly concluded that the intimal lesions were of graft origin (Geraghty et al., 1996). The origin of the cells in allografts was first studied in the sixties and seventies when host repopulation of endothelium was described (Williams and Alvarez, 1969). Later investigations have implicated also non-inflammatory cells originating from the host animal in neointima development in transplants. Thus, analysis of neointimal cells with alloserum demonstrated a discrepancy between the amount of host cells and leukocytes (Plissonnier et al., 1995). This observation was strengthened by experiments showing that development of transplant arteriosclerosis can be reduced by immunosupression, even though the luminal narrowing and SMC proliferation is not necessarily affected (Andersen et al., 1997; Mohacsi et al., 1997; Raisanen-Sokolowski et al., 1995).

Rejection of transplanted organs can be divided into hyperacute, acute, and chronic, dependent on the time of onset. Hyperacute rejection occurs when the recipient has circulating anti-human leukocyte antigen (HLA) antibodies against the donor.

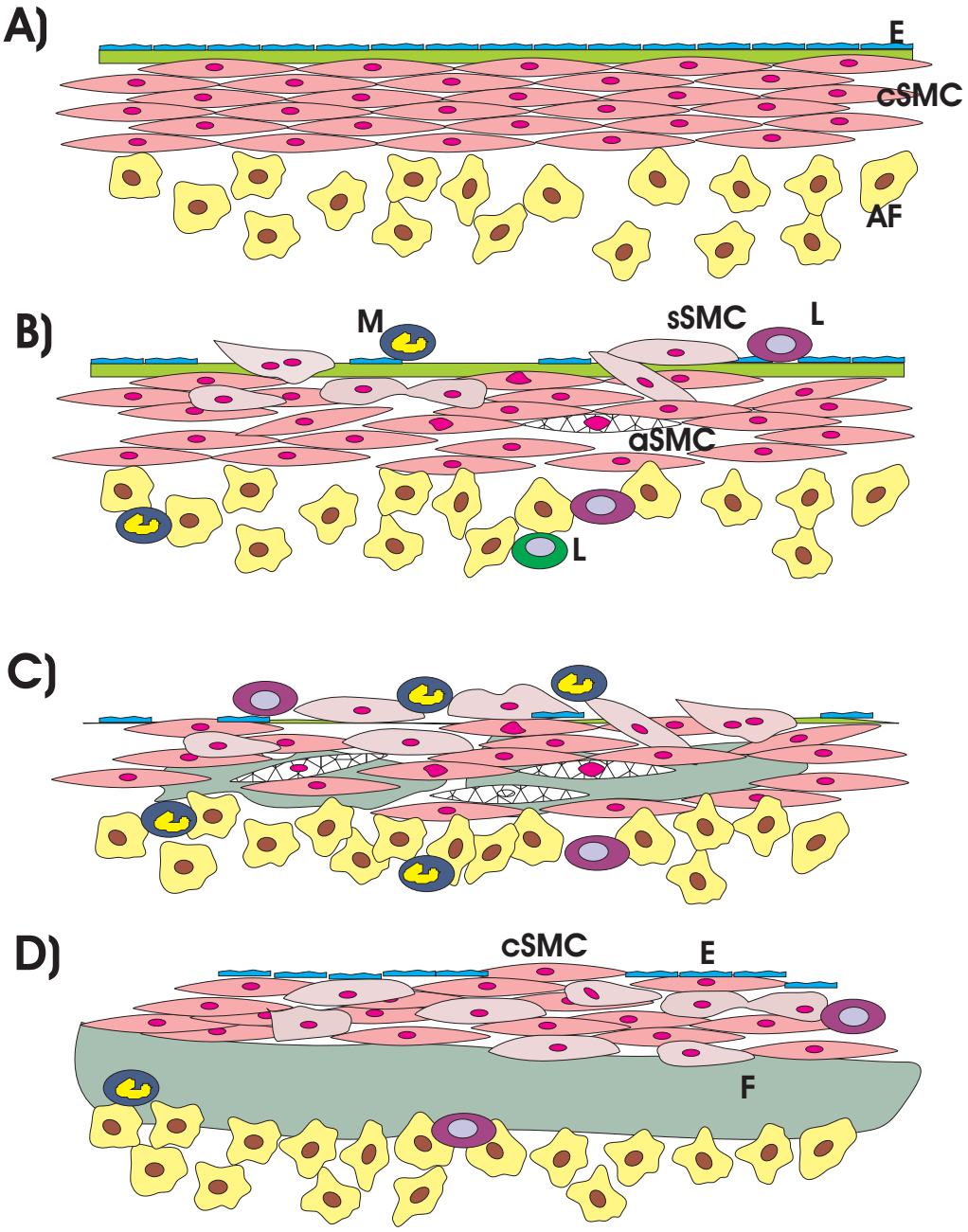

Figure 1. Current understanding of the evolution of transplant arteriosclerosis: A) normal artery (E=endothelial cells; cSMC=contractile SMCs; AF=adventitial fibroblasts), B) endothelial damage, adhesion of inflammatory cells (M=macrophage; L=lymphocyte), phenotypic modulation and migration of medial SMCs to the intima (sSMC=synthetic SMC), and apotosis of SMCs in the media (aSMC=apoptotic SMC) C) formation of intimal hyperplasia by synthetic SMCs and inflammatory cells and progressive apoptosis of medial SMCs, D) further accumulation of SMCs in the neointima, reappearance of endothelial cells on the luminal vessel surface, and progressive fibrosis of the media (F=fibrosis).

The rejection results in an acute vasculitis at the time of transplantation due to a humoral response with binding of antibodies to the endothelium which causes cellular activation and thrombosis (VanBuskirk et al., 1997). Hyperacute rejection can be avoided by cross matching the recipient serum with donor T lymphocytes (Gallon et al., 2002). In contrast, acute rejection is related to cell-mediated inflammatory responses and occurs if HLA mismatching between the host and the donor is present, and is related to the degree of mismatching (VanBuskirk et al., 1997). The rejection involves accumulation of inflammatory cells around vessels and in the parenchyma of the rejected organs. Activation of T lymphocytes can be dependent on the direct presentation of antigens present in the allografts. The cells can be CD8+ cytotoxic cells, CD4+ cells or CD4+ cells mediating delayed hypersensitivity (Chen et al., 1996). Acute rejection also involves B lymphocytes, an indirect presentation of antigens, and the development of a humoral response (Liu et al., 1993); Fig. 2). Since the principal role of the CD4 molecule is to promote interaction of T cells with class II HLA molecules on antigen-presenting cells whereas CD8 interacts with class I HLA molecules, it is not surprising that both types of T cells are usually present and involved in the rejection, what was for example shown in experiments using anti-CD4 and -CD8 antibodies (Nagano et al., 1998). Finally, cytokines such as interleukins (IL-1, IL-2, IL-3, IL-4, IL-6, IL-10, and IL-12), tumor necrosis factor α (TNFα), and interferons are involved in the control of the alloimmune rejection response (Saleem et al., 1996). The process is also related to innate immunity and components such as macrophages, natural killer cells, neutrophils, and complement factors have been described to be involved (He et al., 2002).

Chronic rejection is a term that started to be used in the fifties in order to describe the slow deterioration of transplanted organs and was initially believed to be dependent on a delayed immunological response (Libby and Pober, 2001). Even though chronic rejection is related to immunity, the major finding in allografts affected by this disease is fibrosis of the parenchyma which has developed over months to years. Since this process is also dependent on non-immunological factors (Tullius and Tilney, 1995), it has recently been proposed that chronic rejection should be described as chronic transplant dysfunction (Libby and Pober, 2001). A number of diverse immunological factors which influence the final development of chronic transplant dysfunction have been identified such as the degree of acute rejection, immunosuppression, and concomitant opportunistic infections related to the susceptibility of a recipient under immunosuppressive therapy such as cytomegalovirus (CMV) infection (Soderberg-Naucler and Emery, 2001). In addition, non-immunological factors like donor age,

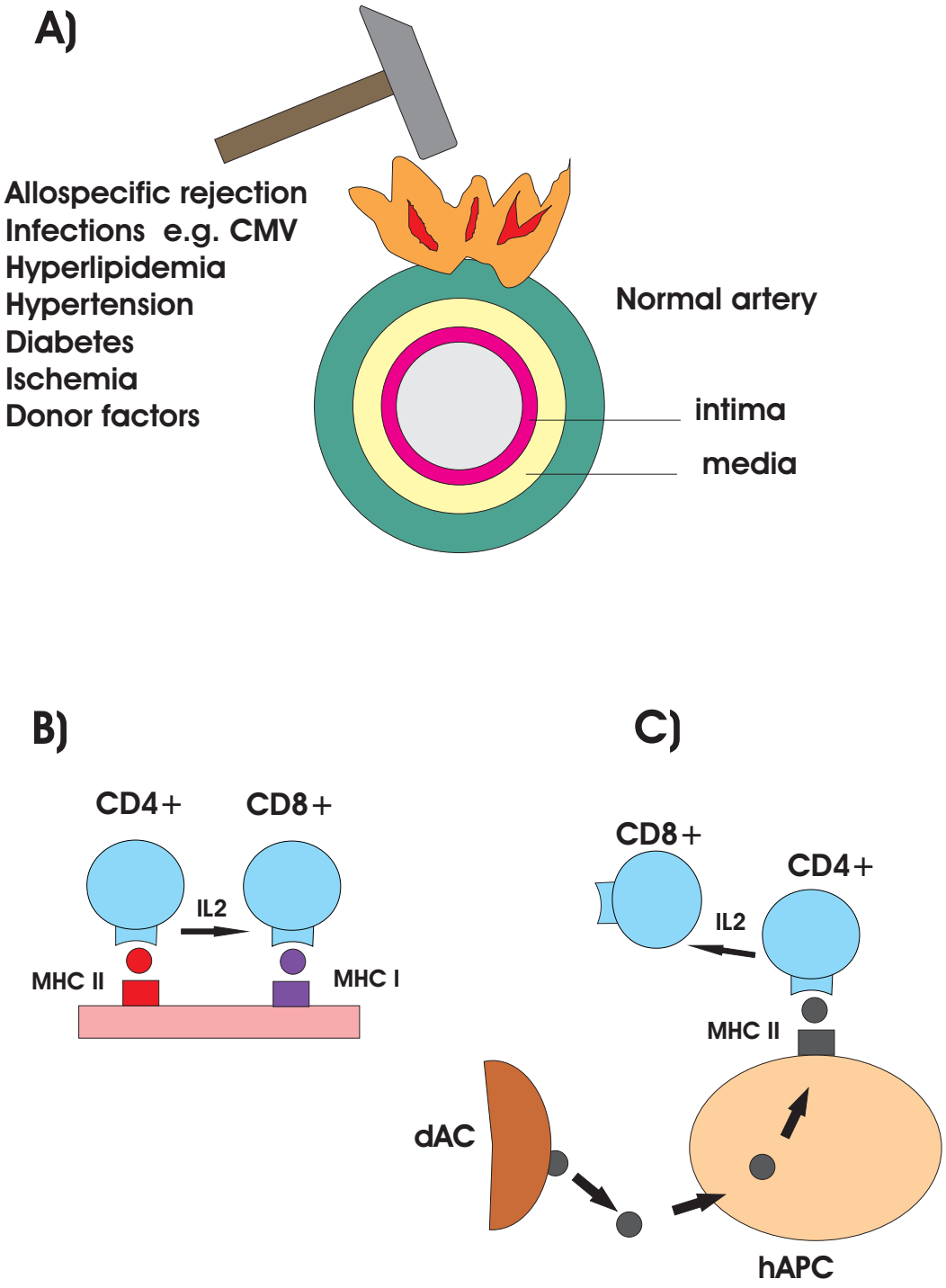

Figure 2. Transplant rejection: A) Factors influencing development of transplant arteriosclerosis, B) Direct recognition occurs when recipient T cells (CD4+, CD 8+) recognize intact donor MHC molecule complexes on donor stimulator cells; C) Indirect recognition occurs when the host antigen presenting cells (APC) process the donor-MHC molecules from donor allogenic cells (dAC) prior to presentation to recipient T cells in a self-restricted manner

quality of the transplanted organ, surgical procedures, and post-transplant factors related to life style and other diseases of the recipient are important (Lu, 1996; Schneeberger et al., 1993). For example, organs from living donors survive better than organs from cadavers (Tullius and Tilney, 1995), and the cause of death of the donor influences the final outcome (Takada et al., 1998). Systemic disease in the donor such as hypertension, diabetes, various infections such as viral hepatitis, hyperlipidemia, and atherosclerosis also affects the survival of the allograft (Fellstrom, 2000).

ATHEROSCLEROSIS

Atherosclerosis is a disease of large and medium-sized elastic and muscular arteries and is characterized by endothelial dysfunction, vascular inflammation, accumulation of lipids and cholesterol, cell death, and calcification within the intima. This process results in the formation of plaques protruding into the vessel lumen, vascular remodeling, acute and chronic luminal obstruction, abnormalities of blood flow, and diminished oxygen supply to the target organs. A complex and incompletely understood interaction exists between endothelial cells, SMCs, platelets and leukocytes of the atherosclerotic lesion. Vasomotor function, the thrombogenicity of the blood vessel wall, the state of activation of the coagulation cascade, the fibrinolytic system, SMC migration and proliferation, and cellular inflammation are complex and interrelated biological processes that contribute to atherogenesis and the clinical manifestations of atherosclerosis (Libby, 2002).

Six separate stages in atherosclerosis have been defined by the American Heart Association (Fig. 3; (Stary et al., 1995). The primary event of atherosclerosis can be described as a biochemical endothelial injury and accumulation of lipids in the subendothelial space. Adaptive changes in the intima, mostly by proteoglycans in the extracellular matrix, followed by infiltration of cells lead to the formation of so called fatty streaks. Activated endothelial cells expressing adhesive molecules, such as vascular cell adhesion molecule (VCAM-1) or intercellular adhesion molecule (ICAM-1), and chemotactic factors, such as macrophage chemoattractant protein-1 (MCP-1), promote adhesion of circulating monocytes. These then enter the intima, become macrophages and take up low-density lipoprotein (LDL) that has accumulated and become oxidized in the intima. A fibrous plaque has developed when accumulation of lipid-filled macrophages and T-lymphocytes forms a lipid core separated from the blood by a fibrous cap generated by SMCs which migrate from the media, proliferate and produce an extracellular matrix rich in collagen (Stary et al., 1995). This process is regulated by several cytokines and growth factors such as transforming growth factor β (TGFβ),

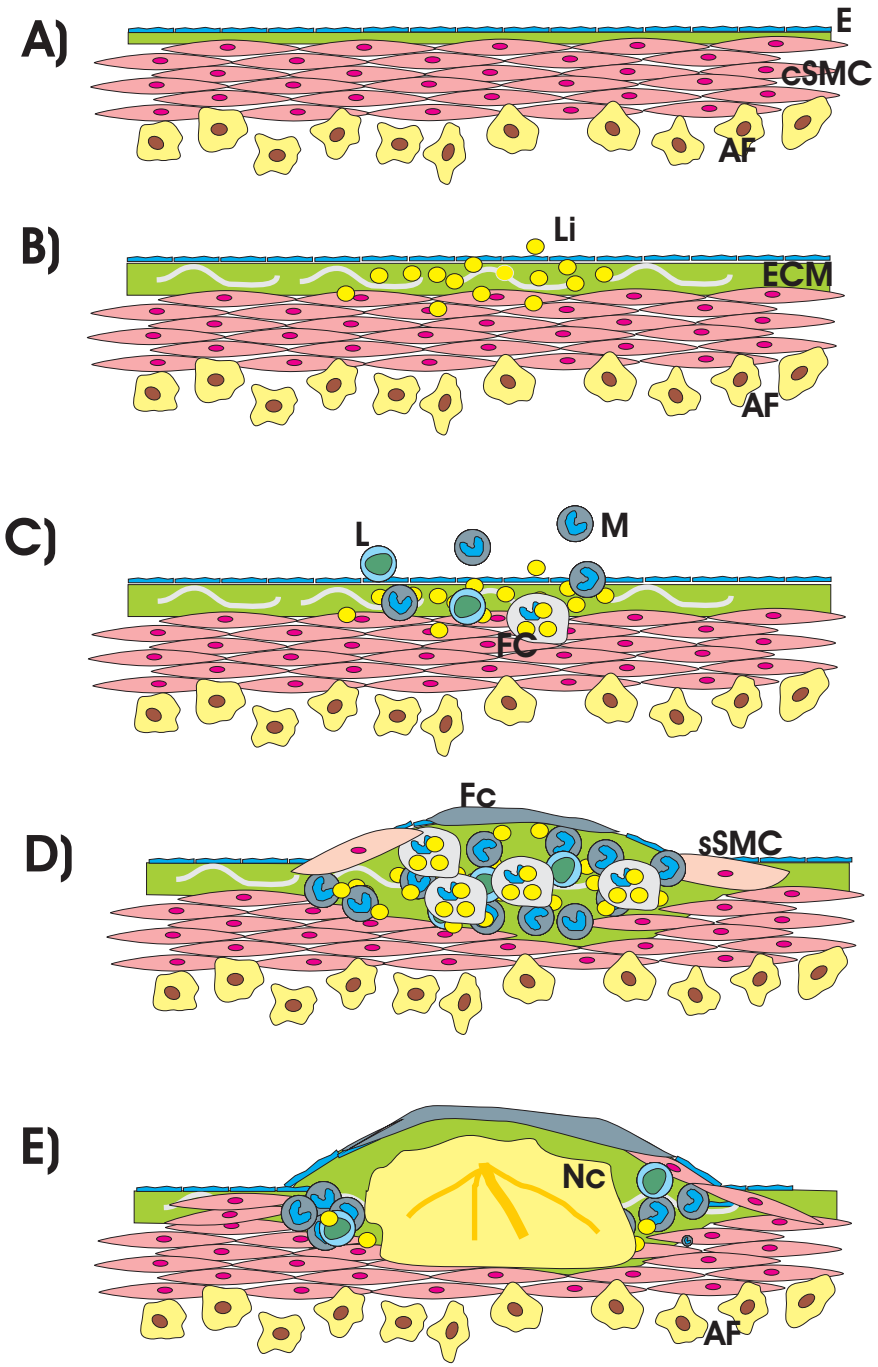

Figure 3. Atherogenesis: A) normal artery (E=endothelial cells; cSMC=contractile SMCs; AF=adventitial fibroblasts), B) infiltration of lipids into the intima (ECM=extracellular matrix), C) formation of a fatty streak in the intima by the infiltration of leukocytes which turn into foam cells by uptake and accumulation of lipid (M=monocyte/macrophage; FC=foam cell; L=limphocyte), D) development of a fibrous plaque with the accumulation of SMCs in a fibrous cap covering lipid-filled leukocytes in the intima (sSMC=synthetic SMC; Fc=fibrous cap), E) formation of an advanced plaque with progressive inflammation, necrosis, and calcification of the lipid core (Nc=necrotic core).

platelet-derived growth factor BB (PDGF BB), and TNFα (Binder et al., 2002; Skoog et al., 2002). The final formation of an advanced atherosclerotic plaque involves further accumulation of inflammatory cells, especially T-cells and macrophages, apoptosis and necrosis of cells in the central part of the lesion (the necrotic core), calcification, and angiogenesis (Stary et al., 1995).

COMPARISON OF TRANSPLANT ARTERIOSCLEROSIS, ATHEROSCLEROSIS AND RESTENOSIS

The structure of the vessel wall changes during development of pathological processes and is related to a balance between all factors influencing the circulation. Factors that participate in this remodeling process include blood flow, shear forces, cell-cell and cell-matrix interactions, injury to vessels by biochemical or physical forces, and inflammatory reactions in the vessel wall (Newby, 2000). Based on experiments with vascular injury in animal models, it was previously believed that remodeling of vessels was limited to reparative processes in the intima related to the death and proliferation of cells. However, it has lately been established that also remodeling of vessel structure in the media and in the adventitia determines luminal narrowing (Glagov, 1994; Hassan et al., 2001). These processes can lead to inward increase in vessel wall thickness with luminal narrowing (negative remodeling) or outward increase in wall thickness without luminal narrowing (positive remodeling). Both negative and positive remodeling can involve intimal hyperplasia, composed mostly of SMCs and extracellular matrix (Fig. 4). Similarities in pathology of transplant arteriosclerosis, atherosclerosis and restenosis after surgical procedures can be seen in Table 1.

INTIMAL HYPERPLASIA

Intimal hyperplasia was first described by Carrel and Guthrie in 1906 when they reported that the anastomis of veins implanted in the arterial circulation was covered with a tissue similar in appearance to endothelium (Carrel and Guthrie, 1906/2001). Although animal models of intimal hyperplasia were introduced in the seventies (Spaet et al., 1975), many questions related to this process remain unsolved. Intimal hyperplasia is defined as an abnormal expansion or thickening of the intima (Schwartz et al., 1975). The process involves activation and phenotypic modulation of medial SMCs, most commonly as a response to physical injury of the vessel, followed by migration of SMCs to the intima (Campbell and Chamley-Campbell, 1981; Thyberg, 1998). Subsequent proliferation of SMCs in the intima with extensive deposition of extracellular matrix gives rise to an intimal lesion that reduces the lumen after endovascular surgery and in bypass grafts (Gruberg et al., 2000). Intimal hyperplasia is also observed in atherosclerosis and in

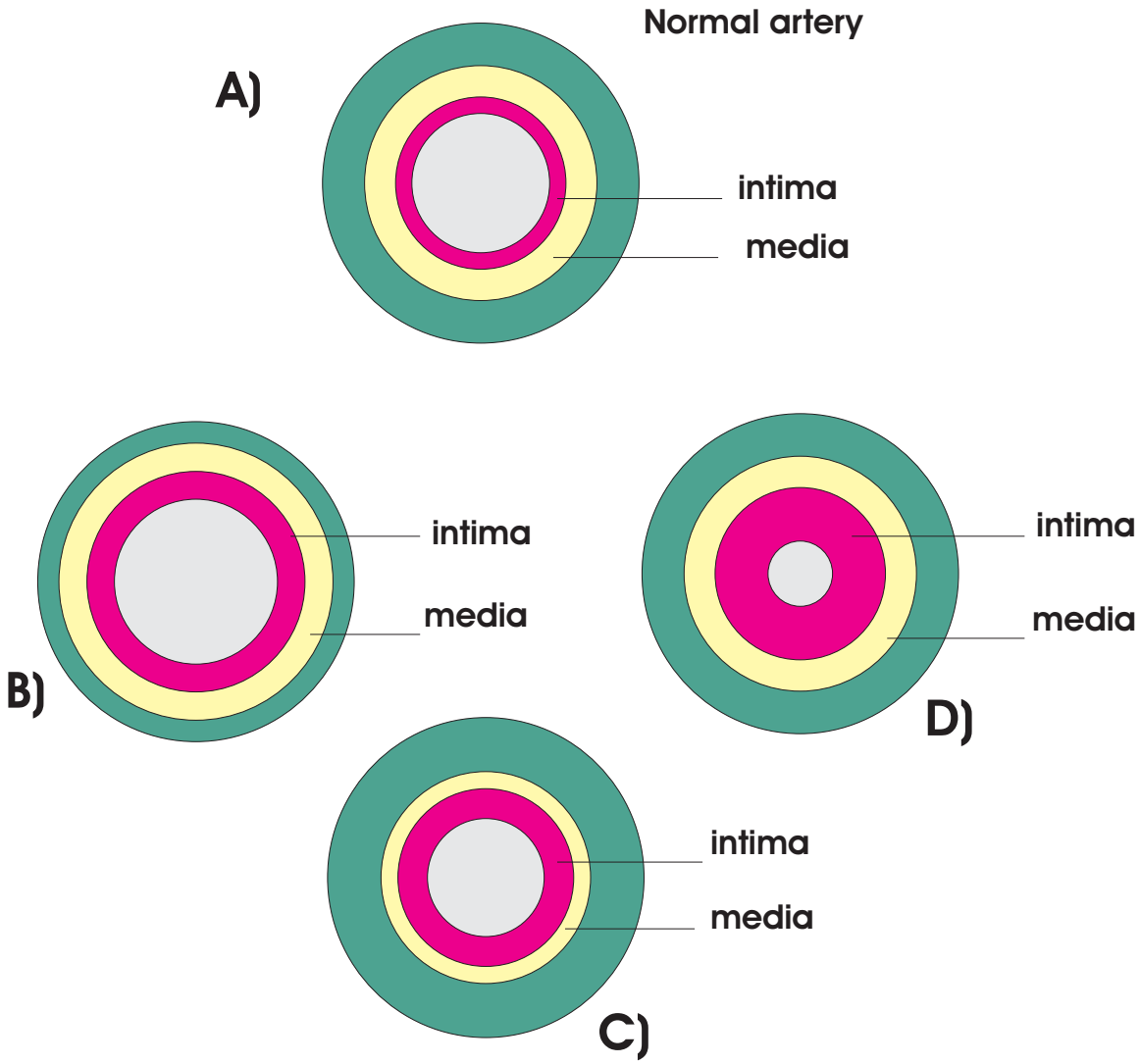

Figure 4. Types of vascular remodeling with formation of intimal hyperplasia: A) normal artery, B) vessel expansion, C) constrictive remodeling, D) intimal thickening

Table 1 Comparison of morphology and events in transplant arteriosclerosis, atherosclerosis and restenosis, according to (Young, 2000) with modification

Characteristic	Transplant arteriosclerosis	Atherosclerosis	Restenosis
Intimal lesion morphology	Concentric, equal distribution	Local, excentric	Local, concentric, according to damage
Vessels involved	All types	Large and medium-sized vessels	Local, related to injury
Initial lesion	SMC proliferation	Fatty streak	SMC proliferation
Internal elastic lamina	Very often intact	Disrupted	Damaged
Cholesterol deposition	Intracellular	Intra- and extra-cellular, local	Usually not present
Endothelium	Very often intact	Disrupted	Disrupted
Leukocytes	Present	Present	Rare
Endothelial MHC II	Present	Absent	Absent
Calcification	Absent, not typical	Present	Absent

transplant arteriosclerosis, at least in part due to triggering by inflammatory stimuli and release of cytokines and growth factors (Geng et al., 1997). Apart from physical injury, hemodynamic factors also induce intimal hyperplasia. Low blood flow and hence low shear stress exerted on the luminal endothelium, not only stimulates the underlying medial SMCs to contraction, but may also lead to vascular remodeling and intimal hyperplasia (Clowes and Berceli, 2000). Ultimately, both processes lead to narrowing of the vessel lumen, increased velocity of the flowing blood and a restoration of shear stress to normal levels (Maalej and Folts, 1996).

Detailed knowledge about the development of intimal hyperplasia has been obtained from the well characterized rat carotid balloon injury model and three phases have been distinguished in the development of intimal hyperplasia in rat (Schwartz et al., 1995). The stages are illustrated in Fig. 5.

Activation and proliferation of medial SMCs

Shortly after deendothelialization of the rat carotid artery by a Fogarty balloon catheter, phenotypic modulation and activation of medial SMCs have been observed by electron microscopy, together with detection of an increased expression of cyclin D1 and an increased synthesis of DNA (Law et al., 1996; Roy et al., 2002). Both migration and proliferation of SMCs are regulated by growth factors. These may be derived from

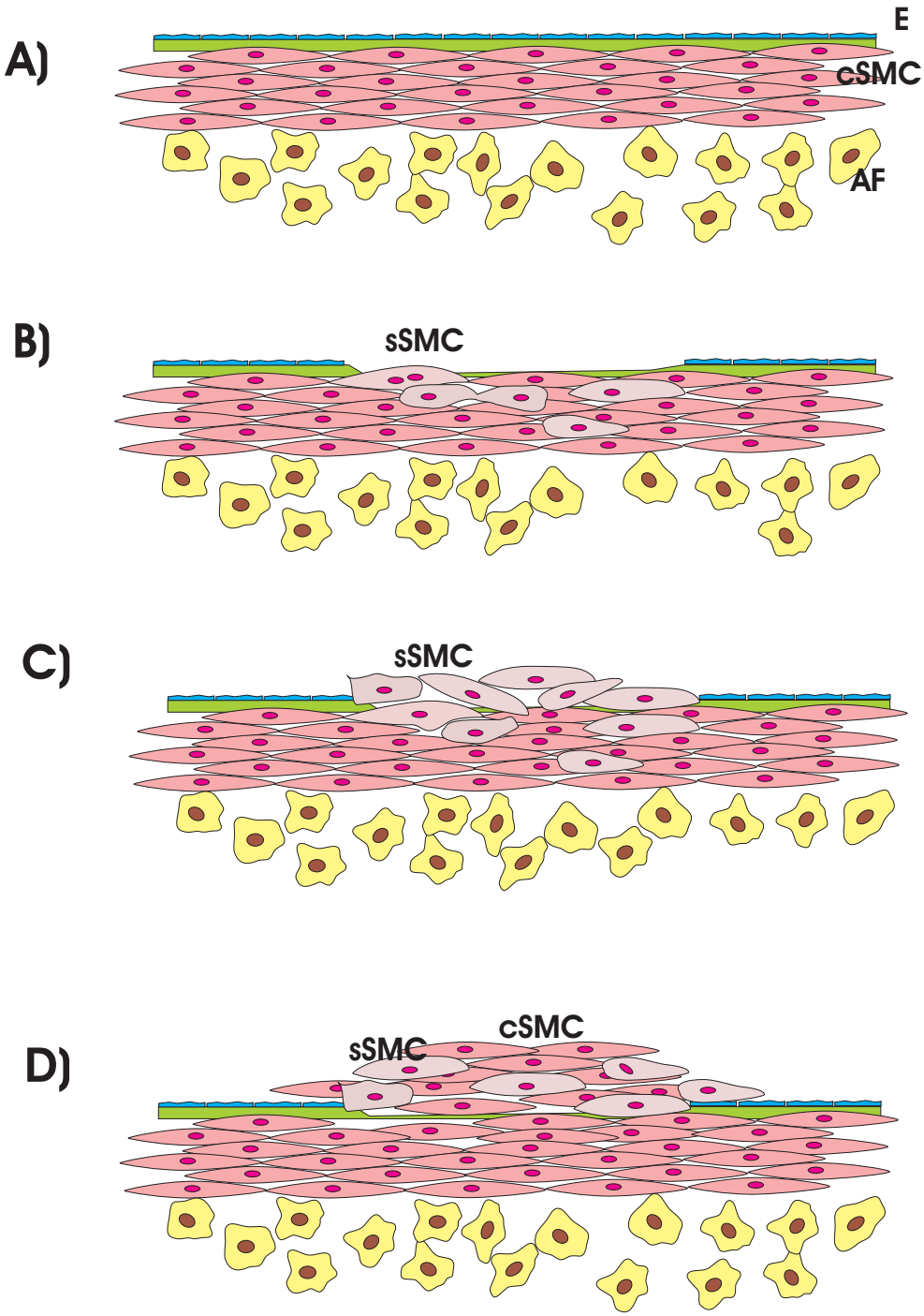

Figure 5. Development of intimal hyperplasia after mechanical injuring of a vessel: A) normal artery (E=endothelial cells; cSMC=contractile SMCs; AF=adventitial fibroblasts), B) deendothelialisation and injury of the media with activation and phenotypic modulation of medial SMCs (sSMC=synthetic SMC), C) migration of synthetic SMCs to the intima, D) formation of an intimal lesion by proliferation and synthesis of extracellular matrix by SMCs.

adhering platelets, leukocytes, and injured endothelial cells or SMCs (Schwartz and Henry, 2002). Especially fibroblast growth factor (FGF) has been identified as an important mitogen for the early phase of SMC proliferation and treatment of injured rat carotid arteries with either FGF antibodies or the FGF-binding polysaccharide heparin prevents the development of intimal hyperplasia (Lindner and Reidy, 1991; Pintucci et al., 1999). However, mitogens are not the only factors involved in SMC activation after injury. For example, exogenously added FGF increases SMC proliferation after vessel injury but does not affect SMC proliferation in uninjured arteries (Lindner and Reidy, 1991; Reidy and Lindner, 1991). This suggests that changes within the vessel related to the injury may also take part in the regulation of SMC activation. After vessel injury, expression of matrix metalloproteinases (MMPs), degradation of laminin and other components in the basement membrane around medial SMCs, and deposition of fibronectin has been suggested to be important for SMC activation (Hedin et al., 1999; Southgate et al., 1996; Thyberg, 1998). This concept is strengthened by the observation that fibronectin promotes SMC activation and cell cycle entry in vitro, whereas laminin prevents activation (Hedin et al., 1988; Roy et al., 2002).

Migration of SMCs

After activation and phenotypic modulation in the media, SMCs migrate to the intima. Although live tracking of migration has never been done due to technical problems, cells crossing the internal elastic lamina are usually visible one week after balloon deendothelialization (Thyberg et al., 1995). SMC migration seems to be stimulated by chemotactic growth factors, such as PDGF BB released from degranulating platelets (Jawien et al., 1992). It has also been shown that endothelial damage induces expression of tissue plasminogen activator and MMPs that facilitate SMC migration to the intima (George et al., 1998).

Formation of the intimal lesion

The first SMCs with a synthetic phenotype are found in the intima about one week after surgery (Thyberg et al., 1995). The intima thereafter grows in size due to continuous SMC proliferation for another 2-4 weeks and deposition of extracellular matrix proteins (Clowes et al., 1983). SMC proliferation is believed to be stimulated by growth factors, such as PDGF BB, angiotensin II and insulin-like growth factor (IGF), released from SMCs in an autocrine or paracrine fashion (Thyberg, 1998). After 4-6 weeks, further growth of the neointima is mainly due to matrix synthesis (Chung et al., 2002).

The concept of intimal hyperplasia presented above is under debate since many questions are still not resolved. New findings and concepts in vascular biology may also add to the picture. Characteristic SMC features with respect to morphology, biochemical markers, and reactivity to mitogens and chemotactic factors change during the different phases of intimal development (Schwartz et al., 1995). At an early stage, the SMCs have a synthetic phenotype with an extensive endoplasmic reticulum and Golgi complex and respond well to growth factors (Thyberg et al., 1995). Later, after 4-6 weeks, the cells become more mature and contractile, with expression of SMC markers such as SM myosin heavy chain, desmin, calponin and SM22α (Halayko et al., 1996). These variations in the characteristics of SMCs can be explained by an adaptation to different physiological conditions by differentiation or dedifferentiation (Schwartz et al., 2000). However, there are also other possible explanations. The most intriguing new concept of vascular biology is that the cells of the neointima are of different origins. For example, it has been shown by the sequential appearance of cell-specific markers and the labeling of adventitial fibroblasts that adventitial fibroblasts can migrate to the neointima (Zalewski and Shi, 1997). In addition, studies by Plissonier in a rat model of transplant arteriosclerosis have suggested that host cells participate in the formation of intimal hyperplasia in allografts (Plissonnier et al., 1995). These findings can be explained by the migration of adjacent host SMCs toward the graft. Another possibility is that circulating cells invade the graft intima and peripheral blood has indeed been reported to contain endothelial- and fibroblast-like precursor cells of bone marrow origin (Asahara et al., 1997; Bucala et al., 1994).

SMOOTH MUSCLE CELLS

SMCs are normally the sole cells of the thickest layer of the vessel wall, the tunica media. They are spindle-shaped and closely associated in circular bundles or sheets. Their boundaries are difficult to distinguish, even though the individual cells are surrounded by a basement membrane (Ross et al., 1995). SMCs are responsible for vessel contraction and relaxation, and so the regulation of blood flow. The SMCs are intimately connected by gap junctions that allow exchange of ions and make the cells function as a syncytium. When one part of the muscle layer is stimulated, the action potential thus spreads throughout all SMCs (Ross et al., 1995). A single nucleus is present in the thickest part of the cell and the cytoplasm is homogenous with numerous myofibrils, a small endoplasmic reticulum and Golgi apparatus, usually plenty of glycogen particles, long slender mitochondria and ribosomes orientated around the nucleus (Ross et al., 1995). The contractile apparatus is composed of SMα-actin, SM myosin and SM tropomyosin.

A number of other cytoskeletal proteins, some also involved in contraction, distinguish SMCs from other cell types, such as calponin, SM-22α, caldesmon, vinculin, metavinculin, desmin, and smoothelin (Sobue et al., 1999). The contraction process is similar to that of skeletal muscle cells, except that SM myosin only interacts with SMα-actin when it has been phosphorylated (Ganong, 2003). Thick myosin and thin actin filaments slide against each other to produce contraction. The filaments are anchored in cytoplasmic dense bodies and dense plaques associated with the plasma membrane. Calcium is stored in the sarcoplasmic reticulum and its mobilization from here and subsequent contraction can be activated by neuronal or hormonal stimulation or by local factors. Neuromuscular junctions are not present and neurotransmitters are instead released from free nerve endings and diffuse around the vessels. Examples of such factors are norepinephrine, histamine and nitric oxide (Fuster et al., 1996; Ganong, 2003).

Despite their sophisticated function in normal vessels, SMCs are not in a terminally differentiated state and have the capacity to regenerate and proliferate. SMC regeneration is related to an overall change in structure and function, often referred to as a phenotypic modulation. SMCs in the media of normal, adult vesssels have a contractile phenotype characterized by quiescence, low synthetic activity and contractile properties (Thyberg, 1996). Both in vivo and in vitro, it has been shown that SMCs can also adopt a synthetic phenotype. This process includes a reduction in the number of myofilaments and a concomitant increase in the amount of synthetic organelles (Fig. 6). There is a decreased expression of cytoskeletal proteins characteristic for contractile cells such as SMα-actin, myosin, vinculin, and desmin (Sobue et al., 1999). Functionally, the cells enter the cell cycle and acquire the capacity to proliferate in response to mitogens and increase their protein synthesis (Fuster et al., 1996; Roy, 2001; Roy et al., 2002). Modulation of medial SMCs from a contractile to a synthetic phenotype is observed during the formation of atherosclerotic plaques, vascular narrowing by formation of a neointima after surgical interventions, and in transplant arteriosclerosis (Thyberg, 1998). In vivo, this process has been studied in detail in connection with intimal hyperplasia after balloon injury of the rat carotid artery (Thyberg et al., 1995). In this model, it has been shown that medial SMCs are activated, lose most of their contractile apparatus and display an increased volume of synthetic organelles. This structural reorganization seems to be required before DNA synthesis and cell division starts (Clowes et al., 1983). After one or a few rounds of proliferation, the cells migrate through the internal elastic lamina and form the first layer of the neointima. Later, the proliferation of these cells and secretion of

extracellular matrix components produce a further growth in size of the neointima. Most likely, the process is regulated by a balance between extrinsic factors and the local environment. It depends on interactions between SMCs, endothelial cells, platelets, mechanical forces, neuronal effects, growth factors, inflammatory cytokines, coagulation factors, and various lipid products (Fuster et al., 1996). Several growth factors which regulate SMC proliferation have been assigned important roles in different vascular pathologies such as PDGF, FGF, TGF, and IGF. These factors are either released from other cell types, such as macrophages, platelets, and endothelial cells, or produced by the SMCs themselves (Chabrier, 1996).

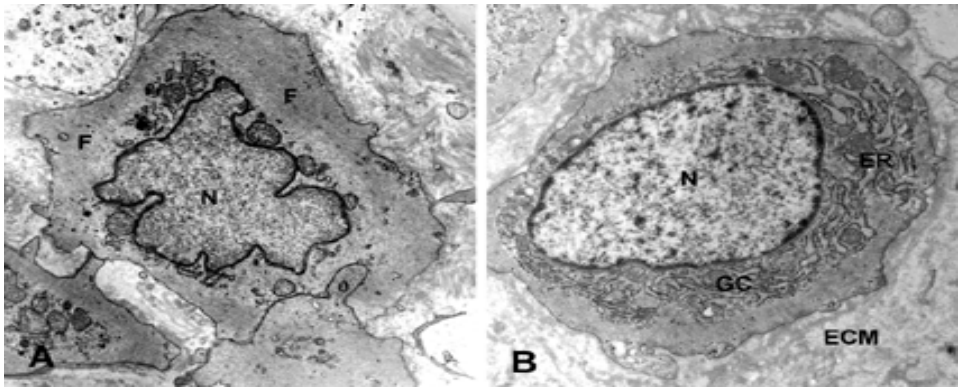

Figure 6. Phenotypic modulation of SMCs in transplanted arteries: A) contractile SMC found in the media of the graft with a heterochromatic nucleus (N), a cytoplasm dominated by myofilaments (F) and sparse synthetic organelles, B) synthetic SMC found in the neointima with an euchromatic nucleus (N), expanded synthetic organelles (GC=Golgi complex; ER=endoplasmatic reticulum) and a reduced amount of myofilaments in the cytoplasm (ECM=extracellular matrix).

SMC PROGENITOR CELLS

Studies in the nineties have shown that organs with terminally differentiated cells such as the brain can regenerate because of the presence of stem cells (Johansson et al., 1999). Moreover, other studies have suggested that an exchange of different cell types take part in the regeneration of organs, as for example shown in the liver (Petersen et al., 1999). Together, these findings have developed a new biological concept with a potential of extraordinary impact for clinical medicine. With respect to vascular biology, a repair process like intimal hyperplasia may also involve stem cells (Rafii et al., 1995). Interestingly, intimal lesions and the vessel wall have for many years been considered to contain SMCs of different phenotypes (Schwartz et al., 2000). This can either be

explained by an adaptation of existing SMCs into different phenotypes (Campbell and Chamley-Campbell, 1981) or indicate that the cells are of different origin (Campbell et al., 2000b).

Stem cells are undifferentiated cells that retain the ability to proliferate throughout postnatal life and so provide a source of progenitor cells that can differentiate into specialized cells in the adult organism. The progenitors can originate from preexisting stem cells as a result of a differentiation process. Alternatively, they can be a result of transdetermination or dedifferentiation (Ying et al., 2002). The first process involves a change of one type of progenitor to another, whereas dedifferentiation refers to a transition of determined cells into progenitor cells and differentiation to other cells (Fig. 7). Pluripotent stem cells such as adult stem cells are found among differentiated cells in tissues or organs and can regenerate and differentiate to yield the major specialized cell types of the tissue or organ (Verfaillie, 2002). In contrast, totipotent stem cells, or embryonic stem cells, have the capacity to specialize into extra-embryonic membranes and tissues, and differentiate into every cell type of the embryo, providing a source for cells to all postembryonic tissues and organs (Dani et al., 1997). The bone marrow is a well known example of an adult tissue compartment with stem cells. Adult stem cells present in the bone marrow can be divided into hematopoietic, mesenchymal, and endothelial stem cells. The first ones are highly active in the generation of erythrocytes and leukocytes. Mesenchymal stem cells form the stroma of the bone marrow which contains a mixture of fibroblasts, reticular cells, osteoprogenitor cells, and multipotent stem cells involved in osteogenesis after bone fractures (Van Damme et al., 2002). A small fraction of bone marrow stem cells expresses endothelial cell markers such as von Willebrand factor (vWf) and takes part in angiogenesis. It has been shown that bone marrow cells can differentiate into SMCs (Galmiche et al., 1993), and endothelial stem cells expressing CD34 and flk 1 have also been demonstrated to differentiate into SMCs when exposed to PDGF BB or fibronectin, both in vitro and in vivo (Pittenger et al., 1999; Yamashita et al., 2000). In fact, the bone marrow seems to be a general source for progenitor cells. For example, bone marrow cells transplanted to mice with myocardial infarction were reported to differentiate into cardiomyocytes (Orlic et al., 2001). Other adult stem cells are mostly found in tissues and organs such as the brain, skin, and interstitium. Lately, it has been found that stem cells have a greater potency than was first believed to differentiate into other cell types and even predetermined stem cells such as neuronal stem cells have the capacity to differentiate into blood cells and myocytes (Bjornson et al., 1999; Galli et al., 2000).

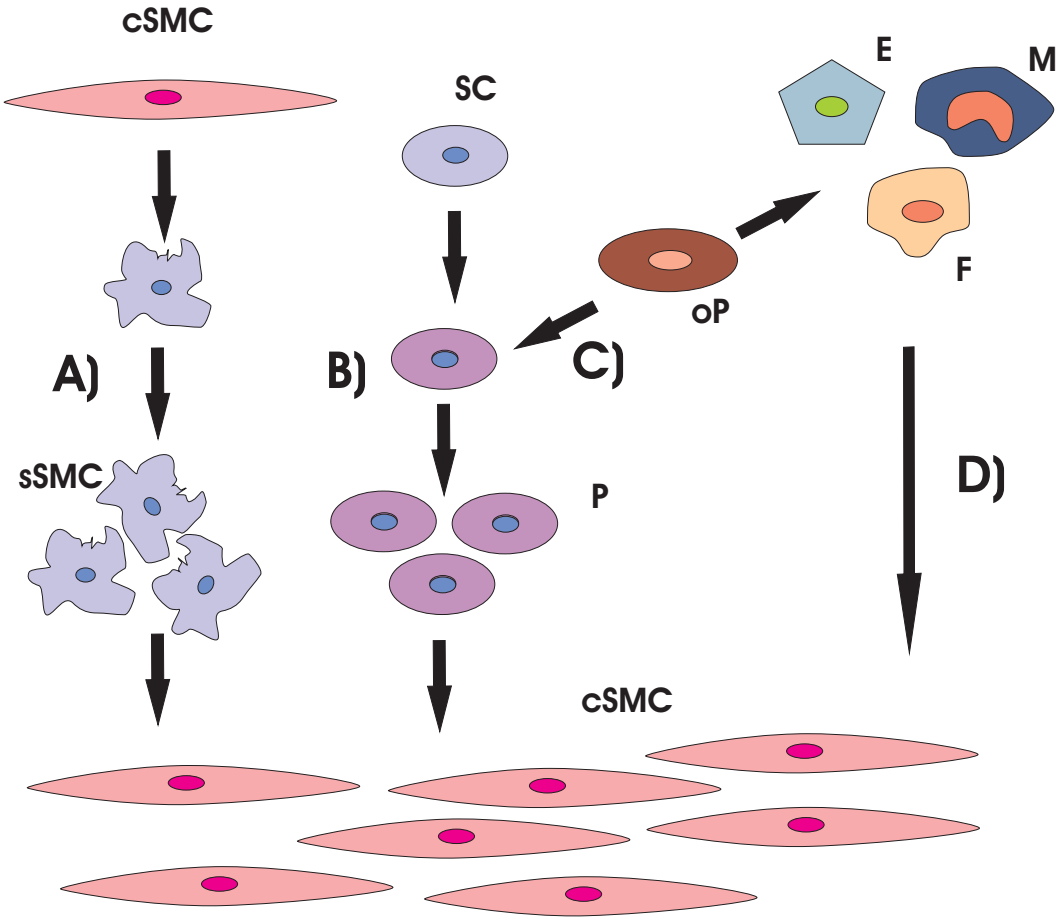

Figure 7. Theoretical sources of SMCs in intimal lesions: A) dedifferentiation of SMCs in the media by phenotypic modulation from a contractile (cSMC) to a synthetic (sSMC) phenotype, B) differentiation from stem cells (SC) and SMC progenitors (P), C) transdetermination of other progenitor cells (oP) such as hematopoietic- or endothelial stem cells to SMC progenitors, D) transformation of other cell types such as macrophages (M), fibroblasts (F), or endothelial cells (E) to SMCs.

The possibility that adult stem cells from the bone marrow take part in tissue repair after injury is a new evolving concept in biology and medicine (Wakitani et al., 1994). The work of Petersen and co-workers has for example shown that bone marrow cells participate in renewal of hepatocytes (Petersen et al., 1999) and a few years after liver transplantation, part of the hematopoietic cells have been shown to be of donor origin (Nierhoff et al., 2000). In addition, hepatocytes and endothelial cells of donor origin have been found in peripheral blood of liver recipients (Korbling et al., 2002). Bone marrow cells have also been demonstrated to differentiate into skeletal muscle and heart muscle cells, and possibly also into neurons (Ferrari et al., 1998; Priller et al., 2001; Stamm et al., 2003). Most of these experiments have been performed by bone marrow transplantation, a model which may introduce artifacts by itself because of the irradiation procedure and the fact that cells spread throughout the body via the circulation (Janczewska et al., 1999). For example, due to the high polymorphism of transplanted cells, it is not possible to distinguish which bone marrow cell line that gives growth of other tissues, or even if it is a natural process since most of the transplanted bone marrow cells remain in the vasculature. Nevertheless, there are only a few other possible explanations for the observed phenomenon such as transformation of macrophages into a specific cell type in the tissue undergoing repair or phagocytosis of inflammatory cells by tissue cells as suggested to occur during intraperitoneal granuloma formation in rabbits (Campbell et al., 2000a). Studies of stem cell biology and related processes such as differentiation, dedifferentiation, or transdifferentiation are strongly dependent on the methodology used (Liu and Rao, 2003). Most of the time, one of the following three methods have been used: (1) labeling of cells in a living tissue with molecular markers and determination of the specialized cell types they generate; (2) removing cells from a living animal, labeling them in cell culture, and transplanting them back into another animal to analyze whether they repopulate the tissue of origin; and (3) isolation and expansion of cells in culture, manipulating them by adding growth factors or introducing new genes, and investigating what differentiated cells types they can give rise to in vitro (NIH, 2002). Cellular labeling can be obtained by using chromosome Y as a marker for cells of male origin, introduction of retroviruses or lipid markers labeled with fluorochromes, or genetic modification of the cells (Cho et al., 2002; Shimizu et al., 2001). In this study, I used the SRY gene in chromosome Y of male cells to follow the infiltration of host cells into the intima of aortic grafts from female rats transplanted into male rats or into female rats with male bone marrow cells.

AIMS

The main objective of this thesis was to explore the cellular mechanisms involved in intimal hyperplasia during transplant arteriosclerosis in the rat aortic allograft transplantation model, with particular emphasis on the biology and function of SMCs in this process. The first paper describes the ultrastructural changes of SMCs that take part in the formation of intimal lesions and the concomitant remodeling of the transplanted aortas. The second paper describes the dynamics of SMC proliferation and apoptosis in relationship to the infiltration of inflammatory cells and changes in the structure and composition of the extracellular matrix in the transplanted vessel. Based on observations in this work and previous reports (Orosz and Pelletier, 1997; Plissonnier et al., 1995) we proposed that recruitment of host SMCs participate in the formation of the neointima, a hypothesis that was further explored in paper 3. The results of the first three papers indicated a correlation between the degree of inflammation and proliferation, apoptosis and accumulation of host-derived SMCs in the intimal lesions of allografts. In the last paper, the mechanisms involved in the accumulation of host SMCs in the lesions were investigated further. The results of this work suggested that the accumulation of host SMCs in intimal lesions is dependent on an allogenic immune response.

MATERIALS AND METHODS

TRANSPLANTATION PROCEDURES OF RAT AORTA

A rat-aorta transplant model was used to perform the study. Briefly, the infrarenal abdominal aorta was transplanted from rats of the F344.RT1v1 strain to rats of the Lewis.RT1 strain (Fig. 8A). The animals were anesthetized with Hypnorm/Diazepam and 1.5-2 cm of the abdominal aorta in F344 rats was removed and washed in 0.9% NaCl. The abdominal aorta was thereafter transplanted orthotopically into Lewis or F344 rats, as allografts or isografts, respectively, using a surgical microscope. This technique was first described as a model of transplant arteriosclerosis (Mennander et al., 1991). The degree of antigenicity after transplantation is related to the degree of histoincompatibility between donor and recipient, and the model used here (LEW.RT1 and F344.RT1v1) is characterized by a low level of antigenicity, making immunosupression unnecessary (el Khatib and Lupinetti, 1990). This model was further modified in order to examine the origin of SMCs during the development of intimal hyperplasia. For this purpose, the female aorta from F344 rats was transplanted to male or female Lewis rats with a bone marrow transplant (see below; Fig. 8B, C). Immunosuppression with cyclosporin A was used to study the role of the immune response for the accumulation of host SMCs in the neointima. In addition, isografts were transplanted after prolonged cold ischemia in order to study intimal hyperplasia in the absence of an immunological incompatibility (Waltenberger et al., 1996).

BONE MARROW TRANSPLANTATION

Bone marrow was transplanted from male Lewis to female Lewis rats to obtain chimeras, either by intravenous injection or hind limb transplantation of syngenic bone marrow. The host rats were first irradiated with 8Gy and later received $6x10^7$ bone marrow cells in suspension i.v. or vascularized bone marrow by hind limb transplantation. The hind limb of the donor was amputated at the groin with the femoral and iliac arteries and veins dissected above this level to obtain long vascular stumps for anastomosis. The host hind limb was amputated at the mid-thigh level and the aforementioned donor limb was attached by an intramedullary metallic stent in the femur, and the vessels connected by end-to-end anastomoses with muscles and skin sutured. The protocol resulted in an equivalent number of transplanted bone marrow cells (Janczewska et al., 1999). However, vascularized bone marrow transplantation gave a better repopulation of hemo- and lymphopoiesis than transplantation of bone marrow by infusion. In addition, 99% of the transplanted cells remained in the vasculature after bone marrow transplantation, which

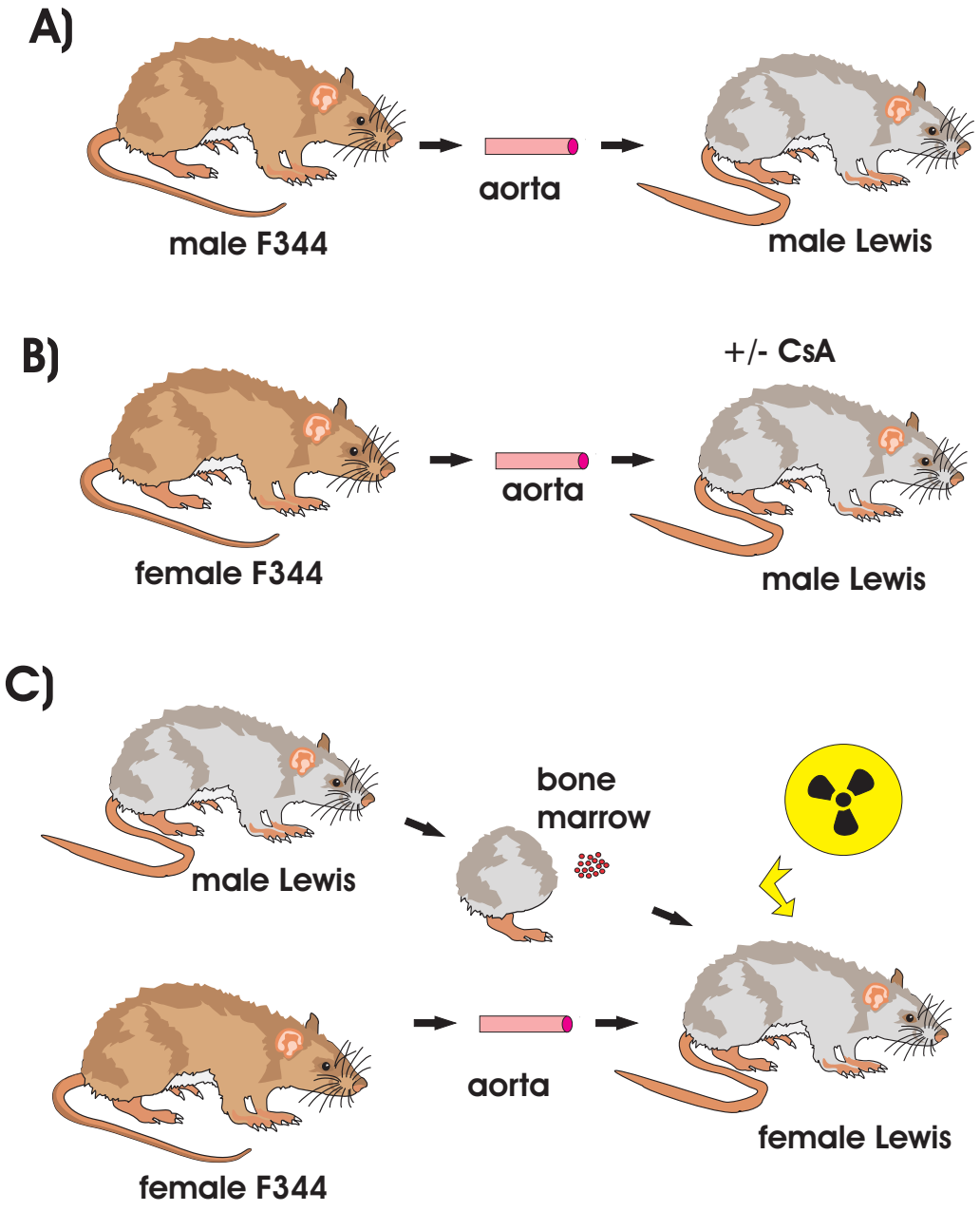

Figure 8. Animal models for transplant arteriosclerosis used in this thesis: A) allograft transplantation of abdominal aorta from male F344 to male Lewis rats, or from male F344 to male F344 rats as isograft controls (paper I, II), B) transplantation of abdominal aorta from female F344 to male Lewis rats, with or without concomitant immunosuppression by cyclosporine A (CsA; paper III, IV), C) transplantation of abdominal aorta from female F344 to female chimerical Lewis rats transplanted with male Lewis bone marrow by infusion or hind limb transplantation after sublethal irradiation.

was a disadvantage compared with vascularized bone marrow transplantation (Lukomska et al., 2000).

RAT CAROTID BALLOON INJURY MODEL

Deendothelialisation of the left common carotid artery of Lewis chimerical rats after bone marrow transplantation was used to investigate if bone marrow-derived cells participate in neointima formation after mechanical injury by denudation of the endothelium using a balloon catheter (Clowes et al., 1983). The animals were anesthetized with Hypnorm/Diazepam and a Fogarty 2F embolectomy catheter was introduced into the left common carotid artery through an arteriotomy in the external carotid artery. The balloon was inflated and passed through the artery three times followed by ligation of the external carotid artery.

IMMUNOHISTOCHEMISTRY

The principle of immunohistochemistry is to raise an antibody (primary antibody) against a specific antigen and use it as a tool to search for the antigen in tissue sections. The antibody can either be directly conjugated to a marker molecule to allow immediate detection, or it can be detected by another antibody (secondary antibody) which recognizes the primary antibody. In this study, most primary antibodies were non-conjugated and detected with appropriate pre-conjugated secondary antibodies. In brief, immunostaining was performed on 4% buffered-formalin-fixed paraffin-embedded sections. The sections were deparaffinized in xylene, rehydrated in water, soaked in 0.03% H_2O_2 in 70% methanol (to block the background activity of tissues enzymes) and incubated with levamisole when double-labeling was performed. Boiling in citrate buffer was used to unmask hidden epitopes and non-specific and electrostatic interactions were blocked with serum diluted in phosphate-buffered saline (PBS). The sections were incubated with primary antibodies overnight followed by washing in PBS and thereafter incubated with secondary antibodies labeled with biotin or a fluorochrome. Biotinylated antibodies were visualized with ABC-Elite Vector horseradish peroxidase (HRP) or ABC-AP Vector alkaline phosphatase (AP) system from Vector Laboratories (Burlingame, CA), and developed with DAB as substrate for HRP or BCIP/NBT as substrate for AP. When double-staining was performed, the first staining was developed with BCIP/NBT followed by incubation with HRP-conjugated anti-SMα-actin antibodies which were detected with the ABC-Elite Vector/DAB system. After rinsing, the sections were counterstained with Mayer's hematoxyline, methyl green, or Vector red.

DETECTION OF CALCIUM PHOSPHATE

Tissue calcification in the grafts was demonstrated with Von Kossa staining. This procedure is based on a redox reaction between calcium and silver and detects calcium phosphate deposits. The rehydrated sections were incubated with 2% aqueous silver nitrate in Coplins staining jars (Sigma, St. Louis, MO) for 60 minutes under a 60 W bulb. The specimens were rinsed in water and exposed to 2.5% aqueous sodium thiosulfate for 5 minutes. The sections were then rinsed quickly five times with distilled water, counterstained with methyl green, and mounted.

LASER CAPTURE MICRODISSECTION

In order to distinguish the origin of cells in aortas transplanted from female to male rats (male cells originating from the recipient) expressing markers for SMCs, apoptosis or cell proliferation, cells stained positive for SMα-actin, caspase-3, or cyclin D1 were microdissected in histological specimens using a laser capture microdissection (LCM) equipment (LCM, PixCell II System, Arcturus Engineering, Mountain View, CA). The samples were prepared for immunohistochemistry as described above, dehydrated with ethanol followed by xylene and then anhydrous $CuSO_4$ in a dessicator. Approximately 200-400 cells were identified and microdissected. The percentage of host-derived cells among the captured cells was thereafter estimated by RT-PCR for the SRY gene (see below).

REAL-TIME PCR

Real-time PCR (RT PCR) was used for the quantification of host cells among the cells isolated from the neointima. RT PCR reflects the initial amount of the template and is the most specific, sensitive and reproducible methodology available today for detection and quantification of genetic material. The RT PCR system is based on the detection and quantification of a fluorescent reporter present on a molecular probe. This signal increases in a direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emitted in each cycle it is possible to monitor the PCR reaction during an exponential phase, where the first significant increase in the amount of PCR product correlates to the initial amount of target template. Therefore, it is a true measurement of the amount of the substrate used. PCR was first used for the detection of microchimerism by (Tashiro et al., 1996) and later by us (Olszewski et al., 1999). Primers and probes for multiplex RT PCR were designed using Perkin Elmer software (Foster City, CA). DNA was extracted using a commercial DNA extraction kit from Perkin Elmer (Foster City, CA). The SRY-specific probe was labeled with FAM

and the probe for a 'house keeping gene' (angiotensin II) was VIC-labeled. The reaction was performed in multiplex using the 7700 Sequence Detection System (Perkin Elmer). The quantification was performed by experimental determination of the cycle threshold values defined as the cycle at which the fluorescence exceeded 10 times the standard deviation of the mean baseline emission for the earlier cycles. Using the baselines determined for both of the genes, the fraction of the SRY gene with respect to the 'house keeping gene' was found.

IN SITU TUNEL

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) is an in situ method for detection of the 3'-OH ends of DNA exposed during the internucleosomal cleavage of DNA that takes place in the process of apoptosis. Incorporation of labeled dUTP allows detection by immunohistochemical procedures and the labeled apoptotic cells may be visualized by light or fluorescence microscopy. Paraffin embedded sections were rehydrated and digested with proteinase K for 30 minutes at room temperature in order to unmask the DNA. The specimens were then incubated with td-transferase and dUTP mixture labeled with biotin (Boehringer Mannheim, Mannheim, Germany) for 0.5-1 hour at 37°C and then washed. The incorporation of biotinylated dUTP was visualized with avidin-HRP (Vector Laboratories, Burlingame, CA) using light microscopy or with Cy2-streptavidin (KPL, Gaithersburg, MD) using fluorescence microscopy. Staining for light microscopy was detected with DAB and the specimens counterstained with methyl green, whereas sections for fluorescence microscopy were double-labeled by incubation with anti-SMα-actin, followed by detection with Cy-2 labeled streptavidin and Cy-5 labeled anti-mouse IgG. Propidium iodide was used for nuclear counterstaining.

IN SITU DETECTION OF CHROMOSOME Y

Detection of the SRY gene in chromosome Y was used for in situ visualization of host- or BM-derived cells. The technique was based on fluorescent in situ hybridization (FISH) and primed in situ labelling (PRINS). FISH was described for the localization of nucleic acids in tissues by (Pardue and Gall, 1969). This method involves three steps: fixation of the specimen, hybridization of labelled probe to homologous fragments of genomic DNA, and fluorescent detection of the labelled probe. Here, FISH was performed using DNA Detector (KPL, Gaithersburg, MD) with the probe prepared using PCR DNA biotinylation kit (KPL, Gaithersburg, MD). Cy2-streptavidin and propidium iodide were used for staining of probe and nuclei. PRINS is an alternative method where biotin-

labeled nucleotides are incorporated into a SRY-specific primer in situ. The sections were prepared for immunohistochemistry with SMC-specific markers, fixed in 2% formaldehyde, and PRINS was catalyzed with Gold ampliTaq enzyme in a thermal cycler (In Situ System 1000; Perkin Elmer). Immunostaining was detected with Cy5-labeled secondary antibody. SRY staining was performed with Cy2-streptavidin, and nuclei counterstained with propidium iodide. The sections were examined in a Zeiss LSM 510 laser scanning confocal microscope. Male aortas were used as positive controls, whereas female aortas without primers or exclusion of the hybridization step were used as negative controls. The in situ techniques of chromosome Y detection cannot be used for quantitative analysis of cellular origin because of difficulties to estimate sensitivity.

CONFOCAL MICROSCOPY

Laser scanning confocal microscopy (LSCM) is based on point scanning principles. A laser light beam is expanded to make optimal use of the optics in the objective and focused to a small spot by the objective lens onto a fluorescent specimen. The reflected light is deviated by a diachronic mirror while the emitted fluorescent light from samples is passed through to the photodetector. A confocal aperture is placed in front of the photodetector to obstruct fluorescent light from points on the specimen that are not within the focal plane. These points are largely obstructed by the pinhole, whereas the focused object enters the center of the pinhole. Here, LSCM was used to obtain high resolution fluorescence images of specimens for chromosome Y detection and TUNEL assay. This technique allowed qualitative visualization of male cells in the allografts and permitted localization of the host-derived cells in the grafts. Images were taken using the Zeiss 510 LSM system fitted with argon/xenon and hell/neon lasers. Pictures were scanned with at least 512x512 pixels resolution, using a 20, 40, or 63x objective and 3 separate tracks for each fluorochrome (Cy2, Cy5, and propidium iodide). Zeiss Vision software was used to optimize the images.

ELECTRON MICROSCOPY

Electron microscopy was used to analyze the ultrastructure of the transplanted vessels with respect to cellular composition, topography and morphology. These studies allowed estimation of the phenotypic modulation of SMCs in the graft media that occured early during the development of transplant arteriosclerosis and processes such as apoptosis, lipid deposition, and calcification. Tissue preparation for electron microscopy involves fixation, dehydration and embedding in low-viscosity epoxy resins that polymerize to give a material suitable for the preparation of ultrathin sections. The main goal of this

process is to stabilize and preserve the fine structural details of the fixed cells as close to that of cells in the living tissues. Glutaraldehyde and and osmium tetroxide (OsO4) were employed as the most effective fixatives for electron microscopy. Glutaraldehyde stabilizes tissues by cross-linking proteins. Osmium tetroxide reacts with lipids and certain proteins but also provides electron density to the tissue. Block staining with uranyl acetate was used to further enhance the electron density of the material. Thin sections were cut with diamond knives on an ultramicrotome. SMCs were analyzed and classified as contractile or synthetic in phenotype according to standards described by (Thyberg et al., 1983). During the phenotypic modulation process, contractile SMCs lose contractile myofilaments whereas the amount of endoplasmatic reticulum and Golgi apparatus (synthetic organelles) increases. The density of different organelles and extracellular structures were determined by point counting morphometry. Cells with condensed nuclei, destroyed organelles and cytoskeleton were classified as apoptotic.

STATISTICS

The percentage of cells among total number of intimal/medial cells was determined by manual counting of cells positive for specific marker as was described in papers II-IV. The neointimal and medial areas were measured with Easy Image Analysis (Bergstrom Instrument, Solna, Sweden). Data were evaluated by analysis of variance (ANOVA) for multiple comparisons among the means. If the ANOVA indicated a significant difference, the individual groups were analyzed by Neuman-Keuls post-hoc test. A significant difference was considered to exist with p-values lower than 0.05.

RESULTS

GENERAL DESCRIPTION

Chronic transplant dysfunction is the single most significant obstacle to long term organ allograft survival. This process manifests itself in a similar manner in all vascularized solid organ allografts as graft vascular disease, obliterative arteriopathy together with interstitial fibrosis and atrophy of parenchymal elements that eventually result in allograft failure. The obliterative arteriopathy leads to vascular narrowing, disturbance in blood flow, ischemia and deterioration of transplanted organs over time (Libby and Pober, 2001). A transplantation model of rat aorta, previously described by (el Khatib and Lupinetti, 1990; Mennander et al., 1991) was used to study this disease process. Briefly, the abdominal aorta was transplanted from F344 rats to Lewis rats. Aortic isografts between F344 rats were used as controls. The grafts were analyzed by light, electron, and confocal microscopy and transplant arteriosclerosis was found to be fully developed in the allografts after 12 weeks of observation and all stages of the disease were found. The investigation was done in order to explain the dynamics and cellular mechanisms behind the progression of the disease.

The study identified four stages of transplant vascular disease, leading to a complete vascular remodeling as typically observed in allografts and previously reviewed in the literature (Labarrere et al., 2001). Early after the transplantation loss of endothelial cells at the luminal surface of the graft was observed. This was followed by activation and proliferation of medial SMCs, leading to the development of intimal lesions. Progression of transplant arteriosclerosis was found to include vessel remodeling with destruction of medial SMCs, deterioration of vessel wall structure with fibrosis and calcification of the media, but also with signs of vessel wall repair by newly recruited SMCs and endothelial cells. The description of the results and the discussion of them are presented in four sections that are related to the chronological stages of disease development and based on papers I-IV.

EARLY CHANGES IN THE STRUCTURE OF AORTIC ALLOGRAFTS

ENDOTHELIAL DAMAGE

The early changes in transplant vascular disease included endothelial damage and activation of medial SMCs. The endothelium covers the internal wall of vessels and regulates vasoconstriction, vasodilatation, hemostasis, and homeostasis of SMCs (Becker et al., 2000). As was observed by electron microscopy and staining for vWF, endothelial damage occurred both in allografts and isografts, but the damage of allografts was more

prominent and included endothelial cell necrosis with organelle damage and degenerative changes (paper I, II). Since the changes were observed both in allografts and controls, they could have been due to factors related to the surgical procedure, for example ischemia. Previously, ischemia has been shown to lead to neutrophil recruitment in cardiac transplants and increased endothelial permeability, a process in which reperfusion may also be involved (Ali et al., 1998; Pinsky et al., 1996). However, regular surgical procedures have not been shown to induce graft disease (Furukawa et al., 2002), whereas prolonged ischemia, even in isografts, causes tissue damage and intimal hyperplasia (Waltenberger et al., 1996); paper IV). Even if the surgical procedure may lead to endothelial injury, immunological factors are necessary for the progression of transplant arteriosclerosis (Stoica et al., 2002). For example, the work by (Rose, 1998) demonstrated that T allo-lymphocytes can be activated by endothelial cells, which thus might be involved in antigen presentation and induction of an allogenic response. In our experiments, a progressive loss of endothelial cells was observed until 4 weeks after the transplantation. Later, a reendothelialisation took place and after 8 weeks, the lumen of the grafts was again covered by endothelial cells (paper I, II). Since the endothelium can both stimulate and inhibit SMC function by factors such as nitric oxide, endothelins, PDGF-BB, FGF or TGFβ (Chabrier, 1996), it is possible that the loss and recovery of endothelial cells also took part in the regulation of intimal lesion development. Previously, eendothelialisation has also been shown to inhibit intimal hyperplasia after vascular injury (Luscher and Tanner, 1993; Powell et al., 1996).

ACTIVATION OF SMCS

Analysis by electron microscopy revealed signs of SMC activation in the media early after transplantation (paper I). SMCs in the inner part of the media changed from a contractile to a synthetic phenotype with reorganization and loss of myofilaments and a progressive expansion of the endoplasmic reticulum and the Golgi complex in a similar manner as previously observed in balloon-injured rat carotid arteries (Thyberg et al., 1997). At the same time, an increased number of SMα-actin positive cells were found in the adventitia adjacent to the outer part of the media, as shown by electron microscopy and immunohistochemistry, suggesting formation of myofibroblasts. Occasional SMCs were also observed in the state of penetrating the internal elastic lamina, indicating SMC migration into the intima. Previously, similar observations have been made after transplantation of the carotid artery (Hamano et al., 1998). (Schwartz et al., 1995) described three phases in the development of intimal hyperplasia after vascular injury: activation of medial SMCs, migration of SMCs into the intima, and formation of intimal

lesions. The activation of SMCs included a shift from a contractile to a synthetic phenotype and cellular proliferation (Nagai et al., 2001). Here, we confirmed SMC activation and phenotypic modulation by electron microscopy and demonstrated a decreased immunostaining for SMα-actin and an increased staining for cellular retinol binding protein-1 (CRBP-1) in SMα-actin-positive cells 2 weeks after transplantation (Fig. 9; (Neuville et al., 1997) have shown that certain clones of medial SMCs express CRBP-1 and believed that these clones might be derived from cells that migrated into the intima and proliferated in connection with vascular damage. In contrast, results by (Roy, 2001) indicated that increased expression of CRBP-1 occurred in cells cultured on fibronectin, a plasma protein that might invade the wall of the aorta after grafting and endothelial damage. These results rather suggest that expression of CRBP-1 is coupled to SMC activation.

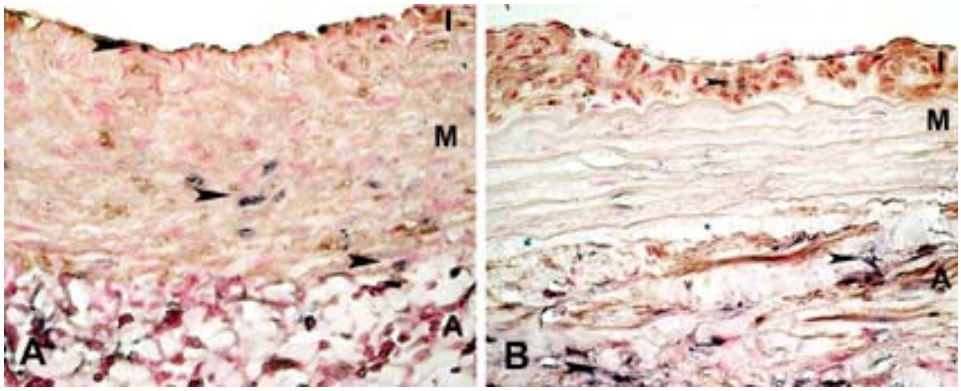

Figure 9. Immunohistochemical double-staining of allografts for SMα-actin (brown) and CRBP1 (dark blue/black) in A) 1- and B) 8-week-old rat aortic allografts (I=intima; M=media; A=adventitia). Positively stained cells are marked by arrowhead.

Proliferation is another marker of SMC activation, and can be verified by immunostaining for cyclinD1 and PCNA as described by (Roy et al., 2002). The cells in the media started to proliferate at week 1-2 (paper I, IV). Contractile SMCs reside in the G0 phase of the cell cycle and need to change phenotype to be able to proliferate and migrate (Roy et al., 2002). As shown by double staining for SMα-actin on one hand and PCNA, cyclin D1, and CRBP-1 on the other hand, activation and proliferation of medial cells was specific for SMCs and was found in the same area of the vessel where signs of SMC activation and phenotypic modulation was observed by electron microscopy (paper I, IV). Even though signs of an intensive proliferation of medial SMCs were observed in our studies, it did not result in an increased medial thickness. On the other hand, a small

expansion of the tunica media was found 4 weeks after the transplantation, probably due to immigration of myofibroblasts from the adventitia. Conceivably, migration of SMCs to the forming neointima as well as apoptosis of SMCs in the media restricted the increase in cell number in the media. Another possibility is that some SMCs duplicate their DNA in the media without cell division, as was shown in studies of arteries from animals with hypertension (Gordon and Schwartz, 1987). In addition, (Hixon et al., 2000) have indicated that polyploidy commonly develops in proliferating populations of SMCs. It is difficult to directly observe the migration of SMCs to the forming neointima, but recent work by (Hasenstab et al., 2000) showed that labeled SMCs injected into the adventitia can later be found in the neointima of ballon-injured vessels. Moreover, both in vivo and in vitro, SMCs express αVβ1, αVβ3 and α1β5 integrins. These matrix receptors have been proposed to be important for cell migration from the media to the neointima (Ikari et al., 2000; Yee et al., 1998). SMC activation, as indicated by phenotypic modulation and proliferation, was in our studies observed before leukocytes infiltrated the graft. This indicated that the early activation of SMCs was related to non-inflammatory processes, such as a loss of the endothelium. This is in agreement with the notion that endothelial denudation and platelet aggregation are involved in SMC activation after vessel injury (Jawien et al., 1992). Factors that might mediate this response include growth factors (Sjolund et al., 1990), cytokines (Shi et al., 1996), matrix degrading proteases (Galis et al., 1994; Hultgardh-Nilsson et al., 1997), and proteins such as fibronectin penetrating into the vessel wall (Hedin et al., 1988; Thyberg et al., 1997). For example, PDGF-BB released from adhering platelets can be a strong stimulator of SMC migration and proliferation (Jawien et al., 1992). However, using electron microscopy and immunostaining we found few platelets on the luminal surface of the allografts early after transplantation. Proteins in the extracellular matrix, such as fibronectin, might also be involved in SMC activation (Roy, 2001; Thyberg et al., 1997), and here we found subendothelial deposition of fibronectin in the allografts (paper II). Recently, (Coito et al., 2001) likewise reported that expression of fibronectin was related to the migration of leukocytes and a progression of transplant arteriosclerosis. We further observed deposition of osteopontin in the allografts, a protein which previously was shown to be involved in SMC migration both in vitro and in vivo (Baron et al., 2000).

FORMATION OF NEOINTIMA

The first neointimal SMCs were observed by electron and light microscopy 1-2 weeks after the transplantation (paper I, II, IV). They were found to be in a synthetic

phenotype with a prominent endoplasmic reticulum and Golgi complex, and stained positively for CRBP-1 and weakly for SMα-actin (paper II, IV). The neointima had a loose structure with local thrombi and an increasing number of leukocytes over time, mostly ED1-positive macrophages but also some CD8- and CD4-positive lymphocytes. The intimal lesions further contained fibronectin, osteopontin, and IgM and IgG1 immunoglobulins, as well as some cells positive for MHC I and II (paper II, IV).

PROLIFERATION OF SMCS IN THE NEOITNIMA

As was shown by double staining for SMα-actin and cyclinD1 or PCNA, proliferating SMCs were present in the intimal lesions. The rate of proliferation was quite high and the number of dividing SMCs progressed from 1-2% up to 10% 1-4 weeks after the transplantation (paper IV). The role of SMC proliferation in the intima seems to be related to lesion expansion, since the frequency of cellular divisions paralleled the growth in size of the neointima. The dynamics and the level of proliferation in the allografts were similar to what has been found in balloon-injured rat carotid arteries (Wei et al., 1997), even though lower proliferation rates have been described in human cardiac allografts (Salom et al., 1994). This difference could be due to the fact that a more intense tissue remodeling occurred in our model. In vitro and in vivo studies of SMCs in atherosclerotic plaques have also demonstrated very low levels of proliferation (Gordon and Schwartz, 1987). However, it would be very difficult to explain an increased intimal mass unless SMC proliferation occurred. It is also possible that the process of SMC replication is limited in time during a long-term process such as atherogenesis and therefore difficult to detect. Factors that could be responsible for the proliferation of SMCs in the intima were most likely the inflammatory reaction and the infiltration of macrophages (Gordon et al., 1990), a possible source of mitogens such as PDGF-BB (Kaiser et al., 1998).

DIVERSITY OF SMCS

Observations both in atherosclerosis, in balloon-injured rat arteries, and in vitro have previously demonstrated that intimal and medial SMCs are different (Halayko and Solway, 2001). Variations have been found both in morphology, biochemical markers and reactivity to mitogens and chemotactic agents (Bochaton-Piallat et al., 1996; Holifield et al., 1996). Neointimal SMCs have a synthetic phenotype during development of this layer, with a low number of contractile myofilaments and an extensive endoplasmic reticulum and Golgi complex. Moreover, both groups of cells are unique in the expression of at least 80 genes including cytoskeletal proteins, growth factors and

adhesive receptors (Schwartz et al., 2000). However, a small fraction of medial SMCs (up to 10%) have been reported to lack contractile proteins and to show an increased reactivity to growth factors (Frid et al., 1997). SMCs from different parts of the vascular system have also been shown to vary with respect to synthesis of ECM proteins and tissue inhibitors of metalloproteinases (Shi et al., 1999).

The diversity of SMCs can be explained by several theories. The most accepted and well proven of these is that the different properties of medial and intimal SMCs are a result of adaptative changes in SMC phenotype. The theory of phenotypic modulation was formed by Chamley-Cambell and coworkers and describes the ability of SMCs to shift between a contractile and synthetic state both in vivo and in vitro (Campbell and Chamley-Campbell, 1981). Based on the observation of a frequent monoclonality of SMCs in atherosclerotic plaques, (Benditt and Benditt, 1973) put forward another theory suggesting that intimal SMCs are derived from a very small number of precursors in the media. Many studies have further demonstrated a sequential appearance of cellular markers in the adventitia, the media and the neointima. In view of these findings, it was suggested that adventitial fibroblasts may also be a source of cells participating in the formation of neointimal lesions (Zalewski and Shi, 1997). A later study by (Li et al., 2000) using a lac-Z labeling system of adventitial fibroblasts seems to confirm this theory. However, reports using antibodies against smoothelin, a newly identified cytoskeletal SMC protein, do not provide support for this notion (Christen et al., 2001). A final and even more intriguing hypothesis proposes that intimal cells can originate from circulating progenitor cells, by transdifferentiation of flk1/CD31 positive endothelial cells (Yamashita et al., 2000) or macrophages to SMCs (Campbell et al., 2000a). Transplant arteriosclerosis is a unique process that offers multiple opportunities to study the role of both donor cells (resident cells in the graft) and recipient cells in the formation of intimal lesions. (Plissonnier et al., 1995) found an incongruity between the number of leukocytes and host-derived cells in the neointima using a rat aortic transplant model. To further explore the possibility that SMCs in the lesions were of host origin, we used a rat male-female aortic allotransplantation model. The donor rats were female and the recipient animals were male. This made it possible to use the SRY gene present on chromosome Y (male) in combination with immunostaining for conventional SMC markers to detect host-derived SMCs. The neointima from the grafts was harvested after different time points and the number of leukocytes and host-derived cells were estimated by immunohistochemistry and RT-PCR. The findings indicated that the progression of intimal lesion development was related to an increased number of host-derived cells

from 20% after 1 week to 70% after 4 weeks, and was paralleled by an increased infiltration of CD45-positive leukocytes into the intima (paper III). At later periods, up to 8 weeks, the number of host cells remained almost constant whereas the number of leukocytes decreased to 11% at week 8. Further analysis by LCM of cells positive for SMα-actin and subsequent RT-PCR for the SRY gene demonstrated that the intimal lesions were mostly composed of graft-derived SMCs early after transplantation. At later times, host-derived SMCs made up most cells in the neointima (paper III, IV). Additional studies of the grafts by FISH and immunohistochemistry followed by PRINS was performed in order to establish: (1) when host cells started to infiltrate the allograft and (2) if these cells expressed SMCs markers such as SMα-actin and SM myosin heavy chains. FISH showed that the appearance of host cells corresponded to infiltration of leukocytes, but part of the SRY-signal was localized in nuclei of SMα-actin and SM myosin-positive cells (Fig. 10). Results indicating an involvement of host-derived cells in the formation of intimal lesions have also been obtained from other transplantation models using lacZ transgenic animals, in situ hybridization for chromosome Y, immunohistochemistry for MHC, and PCR for SRY (Campbell et al., 2000b; Hillebrands et al., 2001; Li et al., 2001; Sata et al., 2002; Shimizu et al., 2001).

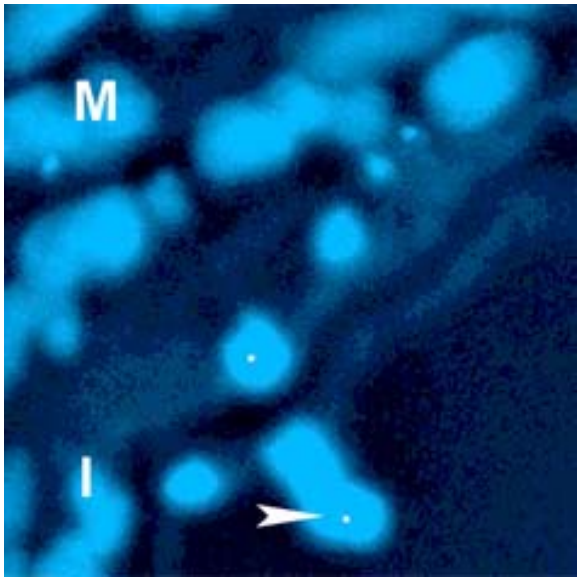

Figure 10. Detection of the SRY gene in chromosome Y in a female rat aorta transplanted to a male recipient by Fluorescent in Situ Hybridization (FISH; M=media; I=intima; arrowhead marks the positive staining in white).

DIVERSE ORIGIN OF SMCS IN FORMATION OF INTIMAL LESIONS

A number of possible sources of host intimal SMCs exist. They could originate from the media or from the adventitia of the adjacent recipient artery wall which migrate into the graft intima.

The host-derived SMCs in the allografts could also represent undifferentiated progenitor cells of bone marrow or tissue origin which are recruited into the graft. It has previously been shown that bone marrow cells can differentiate into SMCs in a mouse myocardial infarction model (Orlic et al., 2001). Moreover, both hematopoietic and mesenchymal stem cells have been shown to differentiate into SMC in vitro (Yamashita et al., 2000). To test this possibility, chimerical rats were prepared by transplantation of bone marrow from male Lewis to female Lewis rats after sublethal irradiation (Janczewska et al., 1999). Since this procedure in itself may generate many artifacts, two different transplantation methods were used; bone morrow transplantation by intravenous injection of purified bone marrow cells and hind limb transplantation which gives a vascularized bone marrow. The last model gives a better bone marrow repopulation after irradiation and does not spread bone marrow cells over the body (Janczewska et al., 1999). Two weeks were allowed for repopulation of recipient bone marrow and recovery of hemopoiesis and lymphopoiesis before allogenic transplantation of female F344 aorta was done. Four weeks later the allografts were collected and the total number of host cells and lymphocytes in the neointima was determined. The structure of the allografts was similar to that in the previously described experiments. However, a variation in the level of tissue destruction was evident and more leukocytes, especially macrophages, were found in allografts from the hindlimb transplantation model, probably due to a better repopulation of the bone marrow and the lymphatic system. Moreover, we found that the total number of host cells in the neointima was higher than the number of leukocytes. For comparative studies, intimal hyperplasia was also analyzed after injury of the carotid arteries and aortas with a Fogarty 2F balloon catheter in the same bone marrow transplanted animals (paper III). In carotids, this procedure resulted in endothelial denudation and medial destruction, and after approximately 2 weeks a prominent neointima was formed, almost exclusively populated by SMCs and with few, if any, inflammatory cells (Clowes et al., 1983). After balloon injury to the aorta, there were few signs of medial destruction and a less prominent neointima was developed (Gabeler et al., 2002). The structure of the neointima after vessel injury in bone marrow transplanted animals was slightly different than what is generally observed in normal animals with more signs of an inflammatory reaction and more necrosis in the media. Possibly, these differences could have been provoked by the irradiation and bone marrow transplantation procedures and a more generalized inflammatory reaction in peripheral tissues (Janczewska et al., 1999). Calculation of host cells by RT PCR indicated that there were up to 10% more host less in the lesions than the number of leukocytes as estimated

by immunohistochemistry. Double staining for chromosome Y/SRY by PRINS combined with immunostaining for SMα-actin or SM myosin also demonstrated neointimal cells that were positive for both of these markers, indicating the presence of SMCs from the host also in the neointima formed after vessel injury (paper III). Previously, it has likewise been reported that bone marrow-derived cells could differentiate into neointimal SMCs in transplant arteriosclerosis or atherosclerosis in transgenic lacZ mice (Shimizu et al., 2001) (Sata et al., 2002). In contrast, studies of transgenic mice with the lacZ-labeled promotor for SM22α did not support this conclusion (Hu et al., 2002a). However, other results from the same group and with the same techniques were somewhat questionable since no activation of the promoter for SM22α was found in SMCs from veins (Hu et al., 2002b). In support of this theory, isolation of cells from human blood and subsequent culture in endothelial cell growth medium supplemented with PDGF BB have also demonstrated the presence of SMC progenitors in the circulation (Simper et al., 2002). There are a few other possible explanations for these findings such as transdifferentiation, transdetermination, and fusion of transplanted bone marrow cells (Terada et al., 2002; Ying et al., 2002). The issue is under discussion and of significant interest both for vascular biology and clinical medicine since the identification of novel sources of neointimal cells may generate new strategies for the prevention of intimal hyperplasia. Recently, endothelial and hemopoietic progenitor cells present in peripheral blood have been termed vascular progenitor cells (Heissig et al., 2002; Hirschi and Goodell, 2002).

Our study provides further support for a possible participation of bone marrow-derived SMC progenitors in neointimal development. However, additional studies are necessary in order to evaluate if this is a normal pathologic process or a phenomenon related to the particular animal model used.

MEDIAL DESTRUCTION AND APOPTOSIS

Apoptosis in aortic allografts was analyzed by electron microscopy, immunostaining for Fas/CD95, caspase-3, and TUNEL assay. The last two analyses were combined with double staining for SMα-actin. Death of endothelial cells was observed shortly after the transplantation both in the isografts and the allografts, but most prominent in the last group. As judged by Fas/CD95 staining and electron microscopy, apoptosis started in the media 1-2 weeks after the transplantation. Some cells were found with condensed nuclei, organelle destruction, formation of apoptotic bodies, and a positive TUNEL staining. There was a discrepancy between Fas/CD95 staining and other apoptosis

markers early after transplantation, possibly due to the fact that separate stages of apoptosis were detected by the used markers (Zavazava and Kabelitz, 2000). However, it has previously been suggested that the Fas pathway of SMC death in atherosclerotic lesions is related to deendothelialisation and induced by Fas ligand derived from monocytes/macrophages (Imanishi et al., 2002). It was recently reported that activated macrophages can rapidly release soluble Fas ligand as an active cytokine, raising the possibility that activated monocytes/macrophages might be able to induce apoptosis in SMCs, even when the two cell populations are located at some distance from each other (Kiener et al., 1997a; Kiener et al., 1997b). Apoptosis might also be enhanced by T lymphocytes and TNFα (Zavazava and Kabelitz, 2000). It has been suggested that apoptosis in transplant arteriosclerosis is the result of a specific immunological targeting of allografts (Lee et al., 2001; Plissonnier et al., 2000), but other factors such as CMV infection and hypercholesterolemia could also be involved (Miller et al., 2001). The maximal apoptosis among SMCs, as judged by caspase-3 and TUNEL staining, was observed 3-4 weeks after the transplantation (paper II, IV). The degenerating SMCs were visible with nuclear condensation and formation of apoptotic bodies, leading to a decreased staining for SMα-actin (paper I, II). In parallel, parts of the media were replaced by fibrosis and formation of foam cells (paper I).

The degenerative changes in the allografts were related to a prominent infiltration of the media and neointima by leukocytes, deposition of immunoglobulins of the IgG1 and IgM classes, and expression of MHC class I and II antigens by many cells. Inhibition of the allogenic immune response with cyclosporin A prevented medial destruction, but did not affect the development of intimal lesions. In support of the finding that apoptosis of SMCs and degeneration of the media was dependent on an immunological reaction, induction of intimal development in isografts by prolonged ischemia, which takes place in the absence of an allogenic immune response, also resulted in intimal lesion development but without medial destruction.

At week 8-12, the final stages of fibrosis in the media, electron and light microscopy together with vonKossa staining demonstrated decreased cellularity, increased amounts of extracellular matrix with collagen fibers, appearance of foam cells, and signs of calcification. Foam cells in apoptotic areas of transplanted vessels have previously been identified as SMCs and macrophages that accumulate cholesterol, possibly derived from apoptotic cells (Granville et al., 1998). Interestingly, apoptosis might even be induced by oxidized derivatives of cholesterol (Haunstetter and Izumo, 1998). Calcification was observed at the border between the media and the neointima and by immunostaining

was found to be related to the depostion of osteopontin. This finding was not surprising since osteopontin has specific functions in tissue calcification (Giachelli and Steitz, 2000), even in atherosclerosis where fibrosis and calcification of the necrotic core represent an end-stage of the disease (Watson and Demer, 1996; Yankah and Wottge, 1997). Although these processes are frequently observed in various animal models of transplant arteriosclerosis, they are rare in human allografts (Young, 2000).

IS THE ACCUMULATION OF HOST-DERIVED SMCS THE RESULT OF AN ALLOGENIC IMMUNE RESPONSE?

The dynamics of accumulation of host-derived SMCs in the neointima indicated that this is a constantantly ongoing process related to the level of medial destruction. The examination of balloon-injured carotid arteries indicated that host-derived SMCs also participate in the formation of intimal lesions after vessel injury, but not to the same degree as in allografts (paper III). The accumulation of host SMCs in the intima could be explained by a constant infiltration of host cells or a faster proliferation of host cells in the allograft intima. Because the first explanation is difficult to study, the latter idea was investigated in the male/female transplantation model. Intimal cells positive for cyclinD1 or caspase-3 were dissected with LCM and their origin was studied by RT PCR for the SRY gene. The results showed that early after transplantation, most proliferating cells were of graft origin, whereas host-derived cells over time made up the major part of the pool of proliferating cells in the intima (paper IV). A similar analysis of apoptotic cells was more difficult to validate since most apoptotic cells were of host origin over the entire 1-8 week period. As mentioned, the level of infiltration of host SMCs as well as inflammatory cells into the intima was more pronounced in allografts than in balloon-injured vessels. In addition, allografts demonstrated multiple signs of an ongoing allogenic immune response with deposition of IgG and IgM, expression of MHC antigens, as well as infiltration of CD4- and CD8-positive lymphocytes (paper IV). These observations suggested to us that the recruitment of host SMCs was related to the level of inflammation and possibly the result of an allogenic immune reaction in the grafts. In order to test this hypothesis, additional experiments were performed in which female F344 aorta was transplanted to male Lewis rats immunosuppressed with cyclosporin A. In parallel, intimal development without concomitant inflammation was analyzed in aortic isografts exposed to prolonged ischemia. The allografts were found to develop a neointima with a similar structure and not significantly different in lesion size from the control allografts, but with less disorganization of the media. In addition, also aortic isografts exposed to prolonged ischemia were observed to develop intimal lesions without medial destruction. Moreover,

the neointima both in isografts and in allografts from immunosuppressed animals had less SMCs of host origin than in allograft controls. Taken together, these findings indicated that an allogenic immune response is involved in the accumulation of host-derived SMCs in intimal lesions during transplant arteriosclerosis. This observation provides new data for the understanding of the development of transplant arteriosclerosis. The concept of removal of graft cells by an immunologically induced process of apoptosis and their subsequent replacement by host-derived cells has not been presented in detail before. The early studies by (Plissonnier et al., 1995) indicated that rat aortic allografts were targeted by an inflammatory response and suggested that part of the SMCs could be of host origin. Later studies by (Geraghty et al., 1996) described the formation of the neointima as a result of phenotypic modulation. Studies by others have further confirmed that the allogenic response is a key factor for development of transplant vasculopathy (Lee et al., 2001; Plissonnier et al., 2000).

GENERAL DISCUSSION AND CONCLUCIONS

The formation of intimal hyperplasia is a significant part of vascular remodeling in transplant arteriosclerosis, atherosclerosis and restenosis following surgical procedures. Animal models of transplant arteriosclerosis offer unique possibilities to examine the formation of intimal hyperplasia in transplanted vessels and to make comparisons with atherosclerosis and restenosis as presented in earlier parts of this summary. Briefly, the intimal lesions in transplant arteriosclerosis have morphological similarities to atherosclerosis, especially with respect to the cell types involved, but the process affects all arteries and lipid cores are not formed (Young, 2000). In addition, the structure of intimal lesions and the following narrowing of the vessel lumen in transplant arteriosclerosis shares feature with restenosis. However, restenosis does not involve medial destruction and the involvement of the immunological system is limited (paper I). It is believed that the mechanisms responsible for the initiation of these disease processes are also different. Transplant arteriosclerosis is mostly related to vascular injury by an allogenic inflammatory response, but other factors that may injure the vessel such as the surgical procedure, hypercholesterolemia and other atherogenic stimuli are also involved (Waaga et al., 2000). In more simplified terms, the complex process of vascular remodeling in transplant arteriosclerosis can be described as an inflammatory reaction in the vessel wall with tissue destruction and repair (Mennander et al., 1993).

THE GENERAL FEATURES OF TRANSPLANT ARTERIOSCLEROSIS

Transplant arteriosclerosis begins with a discrete injury to vessels in the transplanted organ early after the procedure. In this study, the first changes were observed in the endothelial cell layer by electron microscopy and immunohistochemistry for vWF. Since endothelial disintegrity can be found both in allografts and isografts it is likely that these changes are, at least in part, related to the surgical procedure (Lupinetti et al., 1993; Pascual et al., 2002). The following progression of vessel destruction was paralleled by inflammation in the vessel wall with an infiltration by macrophages and lymphocytes, most of all in the adventitia and intima. Later, deterioration of the tunica media with apoptosis of SMCs, reorganization of elastic lamellae, and fibrosis with formation of a collagen-rich extracellular matrix was observed by electron microscopy. Finally, calcification and the appearance of foam cells were found at the border between the media and the neointima. As a tissue reaction, these findings share similarities with the healing of an atheromatous plaque. However, all described components of this process are rarely observed simultaneously in human allografts (Young, 2000).

The study indicated that transplant arteriosclerosis in this model is related to an allogenic immune response against the allograft, which could be modified by immunosuppression. Our results confirm previous observations in the rat aortic transplantation model that cyclosporin A can inhibit aneurysmal dilation of the allograft along with preservation of medial SMCs, delay cellular infiltration, and postpone but not prevent intimal thickening in the graft (Schmitz-Rixen et al., 1988). In addition, previous studies with cyclosporin A have demonstrated that the degree of immunosuppression is related to the level of tissue destruction in transplanted organs (Bigaud et al., 1999). However, since changes were also found in the media of arteries exposed to prolonged ischemia and in balloon-injured carotid arteries in lethally irradiated rats transplanted with bone marrow, medial destruction may not be exclusively dependent on immunological factors (Han et al., 2001; Waltenberger et al., 1996). Taken together, these observations indicate that the extent of medial destruction is dependent on the severity of the injury but not necessarily the type of injury. Both cellular and humoral allogenic responses have been shown to mediate cell death by apoptosis in transplanted organs (Holzknecht et al., 2002). However, apart from immunological factors related to MHC mismatch and imunosuppression, other factors have also been suggested to influence this process such as hyperlipidemia, hypertension, CMV infection, and pre-existing atherosclerosis in the transplanted organ (Radovancevic et al., 1990). The observation that alloserum may induce apoptosis in rat cardiomyocytes indicates that tissue destruction is a specific target for the allogenic immune response (Plissonnier et al., 2000). In addition, it has been shown that indirect recognition of MHC promotes transplant vasculopathy in cardiac allografts in inbred miniature swine (Lee et al., 2001). Taken together, these observations suggest that the allogenic immune response specifically targets the donor cells in the graft, leading to apoptosis of resident SMCs and deterioration of media structure. The healing process in the graft on the other hand, includes fibrosis and calcification in the media, and the formation of intimal hyperplasia.

Formation of a neointima started early after the transplantation in allografts. By electron microscopy, SMCs in a synthetic phenotype were observed both in the inner part of the media and later in the intima 1-2 weeks after the transplantation. In isografts on the other hand, no invasion of SMCs into the intima was observed despite the fact that also these grafts showed signs of deendothelialisation. In contrast, prolonged cold ischemia in isografts resulted in the formation of a neointima. These observations indicate that a permanent deendothelisation with concomitant injury of SMCs in the media, but not necessarily with any involvement of the immune system, leads to the development of

intimal lesions in transplanted vessels, in a similar fashion as during intimal hyperplasia after mechanical injury (Clowes et al., 1983). Intimal lesion expansion was thereafter observed to include accumulation of leukocytes, formation of a loosely structured extracellular matrix. Later, further growth of the neointima was accomplished by the proliferation of SMCs and more deposition of extracellular matrix in a similar manner as previously described (Hancock et al., 1995).

To summarize, transplant arteriosclerosis can roughly be divided into two major phases of vascular remodeling; destruction and healing of the allograft (Plissonnier et al., 1995). Possibly, even a third process related to vessel function should be mentioned since a loss of vasomotor function has been reported to take place in transplanted rat arteries before any morphological changes was observed (Bigaud et al., 1999). In the following, the biological processes involved in the healing phase of transplant arteriosclerosis, the formation of intimal hyperplasia is discussed in more detail.

DEVELOPMENT OF INTIMAL HYPERPLASIA AND THE BIOLOGY OF SMCS

Formation of intimal lesions is a general phenomenon in vascular biology and in vascular disease processes which is almost exclusively dependent on the participation of SMCs. Classically, it is believed that the engagement of SMCs in this process is due to a phenotypic modulation, migration, and proliferation of medial cells (Thyberg, 1998). Later studies have also revealed that activation and phenotypic modulation of SMCs is associated with expression of CRBP1, cyclin D1 and cell cycle entry (Roy, 2001; Roy et al., 2002; Thyberg, 1996). Here, these proteins were found in medial SMCs early after the transplantation and later in the neointima by immunohistochemistry. Similar expression patterns have been described in intimal lesion formation after balloon injury of rat carotid arteries (Neuville et al., 1997; Roy, 2001). Together with the observations we made by electron microscopy, these findings indicated that the process of phenotypic modulation of medial SMCs, with a subsequent migration and proliferation of SMCs was involved in formation of intimal lesion, at least in the early phase of this process.

Apart from the involvement of resident SMCs in the graft media of the donor vessel, previous studies have indicated that also other cells from the adventitia or from the circulation can participate in the generation of intimal lesions in transplanted vessels (Hruban et al., 1993; Plissonnier et al., 1995). In order to study the subject, rat female F344 aortas were transplated to male Lewis rats and the samples harvested after different time points. The presence of host derived SMCs was determined by RT PCR for SRY gene. Our study confirmed that SMCs of recipient animals participate in intimal

hyperlasia and also indicated that the accumulation of host SMCs was related to progressive tissue destruction, especially in the media. The origin, or source, of host-derived SMCs is a remaining question. SMCs may migrate into the graft from adjacent vessels, or they may originate from circulating endothelial- or fibroblast-like cells (Asahara et al., 1997; Bucala et al., 1994). In order to find out if the bone marrow was a source of SMCs, we prepared chimerical female rats with male bone marrow transplanted by infusion or through hind limb transplantation (Janczewska et al., 2000; Lukomska et al., 2000). Later the animals obtained aortic allografts or were exposed to balloon catheter injury of the carotid artery. After both procedures, a neointima containing bone-marrow-derived SM-like cells was formed as determined by immunohistochemistry for SMα-actin and SM myosin combined with RT PCR detection of chromosome Y. This finding is supported by previous observations of SMCs of bone marrow origin both in transplant arteriosclerosis and in atherosclerosis (Sata et al., 2002; Shimizu et al., 2001). In support of this theory, isolation of cells from human blood and subsequent culture in endothelial cell growth medium supplemented with PDGF BB have demonstrated the presence of SMC progenitors in the circulation (Simper et al., 2002). The issue is under discussion and of significant interest both for vascular biology and clinical medicine since the identification of sources of neointimal cells may generate new strategies for the prevention of intimal hyperplasia. Recently, endothelial and hematopoietic progenitor cells present in peripheral blood have been termed vascular progenitor cells (Heissig et al., 2002; Hirschi and Goodell, 2002).

BIPHASIC EVOLUTION OF TRANSPLANT ARTERIOSCLEROSIS

The results presented in paper III indicated that the increased accumulation of host-derived SMCs in the intima corresponded to the level of media destruction which in turn was dependent on an immunological targeting of the transplant (paper I and II). In order to study if an allogenic immune reaction against the allograft participated in the process by removing resident cells from the allograft and/or stimulated the recruitment of host-derived SMCs, immunosuppressive treatment was used. In parallel, apoptosis and proliferation of SMCs as related to their origin (donor or host) was analyzed in female allografts transplanted to male rats. The results indicated that early after the transplantation, the formation of intimal lesions was mainly dependent on SMCs derived from the graft media, in a process resembling intimal hyperplasia after vascular injury. Later, as the destruction of the media progressed, host SMCs infiltrated the neointima and made up the dominating pool of proliferating neointimal cells. A corresponding depletion of resident SMCs by apoptosis was not observed and most apoptotic SMCs in

the neointima were in fact found to be of host origin throughout the time period studied. In support of our initial hypothesis that the accumulation of host SMCs in the allograft depended on the allogenic immune response, both immunosuppression with cyclosporin A and neointima development induced in isografts exposed to prolonged ischemia involved a reduction in the number of host SMCs in the intima. In support of our findings, statins have recently been shown to enhance the release of endothelial progenitor cells from the bone marrow and promote healing of deendothelialised arteries suggesting that the recruitment of vascular progenitor cells into the vessel wall is indeed a process that can be manipulated (Walter et al., 2002). Based on these observations, a novel theory for the development of intimal hyperplasia in transplant arteriosclerosis can be proposed. It suggests that the formation of intimal lesions is biphasic with respect to the removal of resident SMCs from the graft and the recruitment of host SMCs (Fig. 11). This can explain the previously reported early loss and later reappearance of physiological function in aortic allografts (Bigaud et al., 1999). That there are at least two phases in this disease process is also supported by the observations that early inflammatory changes in allografts can be reversed after retransplantation of the graft to the donor stock of animal, whereas the structural changes are not reversible if the retransplantation is performed at a later time (Izutani et al., 1995; Schmid et al., 1996).

It is also important to remember that even if the rat model used in this study shares many features with transplant arteriosclerosis in humans, severe destruction of the media with fibrosis and calcification is rarely observed in human kidney and heart transplants (Libby and Pober, 2001; Young, 2000). The development of transplant arteriosclerosis in this model is based on alloimmunity alone and non-immunological factors previously determined to influence this disease process in clinical transplantology were not considered. Nevertheless, the identification of a possible mechanism responsible for the accumulation of host-derived SMCs in transplant arteriosclerosis may provide a basis for the development of new interventional strategies.

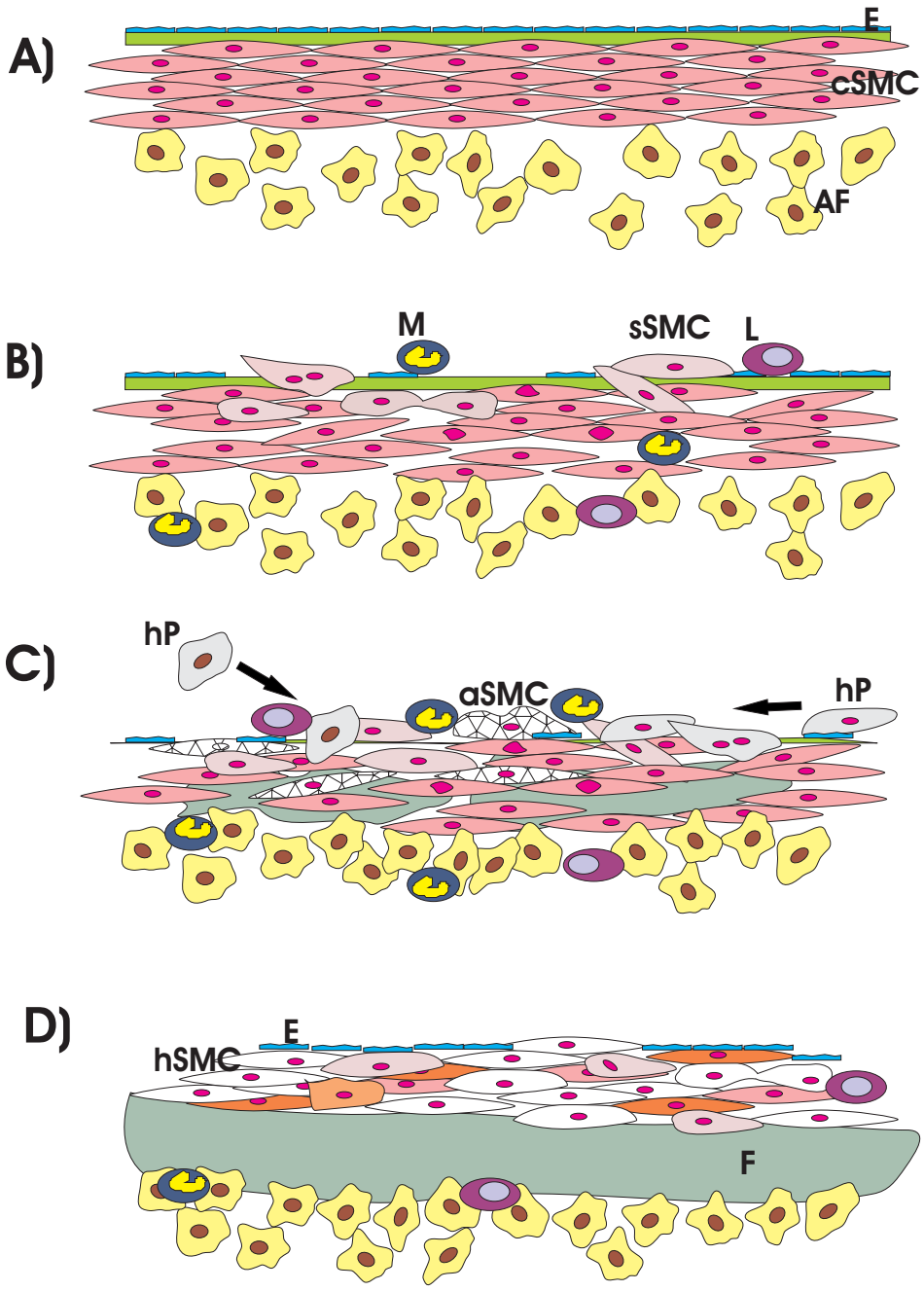

Figure 11. Evolution of transplant arteriosclerosis according to the major findings in this thesis: A) normal artery (E=endothelial cells; cSMC=contractile SMCs; AF=adventitial fibroblasts), B) endothelial damage and the initial formation of a neointima by activation of SMCs in the graft media and infiltration of the graft by inflammatory cells indicating an allogenic immune response (sSMC=synthetic SMC; M=monocyte/macrophage; L=lymphocyte), C), this immunological reaction promotes apoptosis of resident SMCs in the graft media (aSMC) and progressive infiltration of the neointima by host-derived SMC progenitors (hP), D) further accumulation of host-derived SMCs (hSMC) in the neointima, reappearance of endothelial cells at the graft luminal surface, and fibrosis (F) in the media.

FUTURE DIRECTIONS

The concept that host-derived SMCs participate in intimal hyperplasia in transplant arteriosclerosis appears to be well established. However, the number of possible sources of these cells is still large and needs to be further explored. In addition, a better characterization of the diversity of SMCs and the specific properties of these cells not only in transplant arteriosclerosis but also in intimal lesions in general require additional investigations. Much more research also remains in order to learn about the mechanisms involved in the accumulation of host SMCs and what factors influence the proliferation and apoptosis of these cells. The research in this field can generate strategies to inhibit intimal hyperplasia after surgical procedures, in transplant arteriosclerosis, and may also lead to possibilities to monitor the stability of atherosclerotic plaques.

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REFERENCES

Ali, M. H., Schlidt, S. A., Hynes, K. L., Marcus, B. C., and Gewertz, B. L. (1998). Prolonged hypoxia alters endothelial barrier function. Surgery *124*, 491-497.

Amouyel, P. (2002). Genetic susceptibility to ageing-associated diseases. C R Biol *325*, 741-745.

Andersen, H. O., Holm, P., Stender, S., Hansen, B. F., and Nordestgaard, B. G. (1997). Dose-dependent suppression of transplant arteriosclerosis in aorta-allografted, cholesterol-clamped rabbits. Suppression not eliminated by the cholesterol-raising effect of cyclosporine. Arterioscler Thromb Vasc Biol *17*, 2515-2523.

Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G., and Isner, J. M. (1997). Isolation of putative progenitor endothelial cells for angiogenesis. Science *275*, 964-967.

Baron, J. H., Moiseeva, E. P., de Bono, D. P., Abrams, K. R., and Gershlick, A. H. (2000). Inhibition of vascular smooth muscle cell adhesion and migration by c7E3 Fab (abciximab): a possible mechanism for influencing restenosis. Cardiovasc Res *48*, 464-472.

Becker, B. F., Heindl, B., Kupatt, C., and Zahler, S. (2000). Endothelial function and hemostasis. Z Kardiol *89*, 160-167.

Benditt, E. P., and Benditt, J. M. (1973). Evidence for a monoclonal origin of human atherosclerotic plaques. Proc Natl Acad Sci U S A *70*, 1753-1756.

Bigaud, M., Schraa, E. O., Andriambeloson, E., Lobstein, V., Pally, C., Kobel, T., Bruns, C., and Zerwes, H. G. (1999). Complete loss of functional smooth muscle cells precedes vascular remodeling in rat aorta allografts. Transplantation *68*, 1701-1707.

Binder, C. J., Chang, M. K., Shaw, P. X., Miller, Y. I., Hartvigsen, K., Dewan, A., and Witztum, J. L. (2002). Innate and acquired immunity in atherogenesis. Nat Med *8*, 1218-1226.

Bittl, J. A. (1996). Advances in coronary angioplasty. N Engl J Med *335*, 1290-1302.

Bjornson, C. R., Rietze, R. L., Reynolds, B. A., Magli, M. C., and Vescovi, A. L. (1999). Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. Science *283*, 534-537.

Bochaton-Piallat, M. L., Ropraz, P., Gabbiani, F., and Gabbiani, G. (1996). Phenotypic heterogeneity of rat arterial smooth muscle cell clones. Implications for the development of experimental intimal thickening. Arterioscler Thromb Vasc Biol *16*, 815-820.

Bucala, R., Spiegel, L. A., Chesney, J., Hogan, M., and Cerami, A. (1994). Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. Mol Med *1*, 71-81.

Campbell, G. R., and Chamley-Campbell, J. H. (1981). Smooth muscle phenotypic modulation: role in atherogenesis. Med Hypotheses *7*, 729-735.

Campbell, J. H., Efendy, J. L., Han, C., Girjes, A. A., and Campbell, G. R. (2000a). Haemopoietic origin of myofibroblasts formed in the peritoneal cavity in response to a foreign body. J Vasc Res *37*, 364-371.

Campbell, J. H., Efendy, J. L., Han, C. L., and Campbell, G. R. (2000b). Blood vessels from bone marrow. Ann N Y Acad Sci *902*, 224-229.

Carrel, A., and Guthrie, C. C. (1906/2001). Anastomosis of blood vessels by the patching method and transplantation of the kidney. 1906 [classical article]. Yale J Biol Med *74*, 243-247.

Cecka, J. M. (2000). The UNOS Scientific Renal Transplant Registry--2000. Clin Transpl, 1-18.

Chabrier, P. E. (1996). Growth factors and vascular wall. Int Angiol *15*, 100-103.

Chen, W., Murphy, B., Waaga, A. M., Willett, T. A., Russell, M. E., Khoury, S. J., and Sayegh, M. H. (1996). Mechanisms of indirect allorecognition in graft rejection: class II MHC allopeptide-specific T cell clones transfer delayed-type hypersensitivity responses in vivo. Transplantation *62*, 705-710.

Cho, J. J., Malhi, H., Wang, R., Joseph, B., Ludlow, J. W., Susick, R., and Gupta, S. (2002). Enzymatically labeled chromosomal probes for in situ identification of human cells in xenogeneic transplant models. Nat Med *8*, 1033-1036.

Christen, T., Verin, V., Bochaton-Piallat, M., Popowski, Y., Ramaekers, F., Debruyne, P., Camenzind, E., van Eys, G., and Gabbiani, G. (2001). Mechanisms of neointima formation and remodeling in the porcine coronary artery. Circulation *103*, 882-888.

Chung, I. M., Gold, H. K., Schwartz, S. M., Ikari, Y., Reidy, M. A., and Wight, T. N. (2002). Enhanced extracellular matrix accumulation in restenosis of coronary arteries after stent deployment. J Am Coll Cardiol *40*, 2072-2081.

Clowes, A. W., and Berceli, S. A. (2000). Mechanisms of vascular atrophy and fibrous cap disruption. Ann N Y Acad Sci *902*, 153-161; discussion 161-152.

Clowes, A. W., Reidy, M. A., and Clowes, M. M. (1983). Mechanisms of stenosis after arterial injury. Lab Invest *49*, 208-215.

Coito, A. J., Kato, H., Azimi, R., and Kupiec-Weglinski, J. W. (2001). Chronic allograft rejection versus tolerance: a critical role for EIIIA(+) fibronectin. Transplant Proc *33*, 526-527.

Dani, C., Smith, A. G., Dessolin, S., Leroy, P., Staccini, L., Villageois, P., Darimont, C., and Ailhaud, G. (1997). Differentiation of embryonic stem cells into adipocytes in vitro. J Cell Sci *110 (Pt 11)*, 1279-1285.

Dawson, D. L., Hiatt, W. R., Creager, M. A., and Hirsch, A. T. (2002). Peripheral arterial disease: medical care and prevention of complications. Prev Cardiol *5*, 119-130.

Depre, C., Havaux, X., and Wijns, W. (1998). Pathology of restenosis in saphenous bypass grafts after long-term stent implantation. Am J Clin Pathol *110*, 378-384.

el Khatib, H., and Lupinetti, F. M. (1990). Antigenicity of fresh and cryopreserved rat valve allografts. Transplantation *49*, 765-767.

el-Sanadiki, M. N., Cross, K. S., Murray, J. J., Schuman, R. W., Mikat, E., McCann, R. L., and Hagen, P. O. (1990). Reduction of intimal hyperplasia and enhanced reactivity of experimental vein bypass grafts with verapamil treatment. Ann Surg *212*, 87-96.

Fellstrom, B. (2000). Impact and management of hyperlipidemia posttransplantation. Transplantation *70*, SS51-57.

Ferrari, G., Cusella-De Angelis, G., Coletta, M., Paolucci, E., Stornaiuolo, A., Cossu, G., and Mavilio, F. (1998). Muscle regeneration by bone marrow-derived myogenic progenitors. Science *279*, 1528-1530.

Frid, M. G., Aldashev, A. A., Dempsey, E. C., and Stenmark, K. R. (1997). Smooth muscle cells isolated from discrete compartments of the mature vascular media exhibit unique phenotypes and distinct growth capabilities. Circ Res *81*, 940-952.

Furness, P. N. (2001). Histopathology of chronic renal allograft dysfunction. Transplantation *71*, SS31-36.

Furukawa, Y., Libby, P., Stinn, J. L., Becker, G., and Mitchell, R. N. (2002). Cold ischemia induces isograft arteriopathy, but does not augment allograft arteriopathy in non-immunosuppressed hosts. Am J Pathol *160*, 1077-1087.

Fuster, V., Ross, R., and Topol, E. J. (1996). Atherosclerosis and Coronary Artery Disease. Lippincott-Raven Publishers, Philadephia, 386-435.

Gabeler, E. E., Van Hillegersberg, R., Statius Van Eps, R. G., Sluiter, W., Gussenhoven, E. J., Mulder, P., and Van Urk, H. (2002). A comparison of balloon injury models of endovascular lesions in rat arteries. BMC Cardiovasc Disord *2*, 16.

Galis, Z. S., Johnson, C., Godin, D., Magid, R., Shipley, J. M., Senior, R. M., and Ivan, E. (2002). Targeted disruption of the matrix metalloproteinase-9 gene impairs smooth muscle cell migration and geometrical arterial remodeling. Circ Res *91*, 852-859.

Galis, Z. S., Muszynski, M., Sukhova, G. K., Simon-Morrissey, E., Unemori, E. N., Lark, M. W., Amento, E., and Libby, P. (1994). Cytokine-stimulated human vascular smooth muscle cells synthesize a complement of enzymes required for extracellular matrix digestion. Circ Res *75*, 181-189.

Galli, R., Borello, U., Gritti, A., Minasi, M. G., Bjornson, C., Coletta, M., Mora, M., De Angelis, M. G., Fiocco, R., Cossu, G., and Vescovi, A. L. (2000). Skeletal myogenic potential of human and mouse neural stem cells. Nat Neurosci *3*, 986-991.

Gallon, L. G., Leventhal, J. R., and Kaufman, D. B. (2002). Pretransplant evaluation of renal transplant candidates. Semin Nephrol *22*, 515-525.

Galmiche, M. C., Koteliansky, V. E., Briere, J., Herve, P., and Charbord, P. (1993). Stromal cells from human long-term marrow cultures are mesenchymal cells that differentiate following a vascular smooth muscle differentiation pathway. Blood *82*, 66-76.

Ganong, A. (2003). Review of Medical Physiology, McGraw-Hill/Appleton & Lange, 2001.

Geng, Y. J., Henderson, L. E., Levesque, E. B., Muszynski, M., and Libby, P. (1997). Fas is expressed in human atherosclerotic intima and promotes apoptosis of cytokine-primed human vascular smooth muscle cells. Arterioscler Thromb Vasc Biol *17*, 2200-2208.

George, S. J., Baker, A. H., Angelini, G. D., and Newby, A. C. (1998). Gene transfer of tissue inhibitor of metalloproteinase-2 inhibits metalloproteinase activity and neointima formation in human saphenous veins. Gene Ther *5*, 1552-1560.

Geraghty, J. G., Stoltenberg, R. L., Sollinger, H. W., and Hullett, D. A. (1996). Vascular smooth muscle cells and neointimal hyperplasia in chronic transplant rejection. Transplantation *62*, 502-509.

Giachelli, C. M., and Steitz, S. (2000). Osteopontin: a versatile regulator of inflammation and biomineralization [In Process Citation]. Matrix Biol *19*, 615-622.

Glagov, S. (1994). Intimal hyperplasia, vascular modeling, and the restenosis problem. Circulation *89*, 2888-2891.

Gonin, J. M. (2000). Maintenance immunosuppression: new agents and persistent dilemmas. Adv Ren Replace Ther *7*, 95-116.

Gordon, D., Reidy, M. A., Benditt, E. P., and Schwartz, S. M. (1990). Cell proliferation in human coronary arteries. Proc Natl Acad Sci U S A *87*, 4600-4604.

Gordon, D., and Schwartz, S. M. (1987). Replication of arterial smooth muscle cells in hypertension and atherosclerosis. Am J Cardiol *59*, 44A-48A.

Granville, D. J., Carthy, C. M., Hunt, D. W., and McManus, B. M. (1998). Apoptosis: molecular aspects of cell death and disease. Lab Invest *78*, 893-913.

Gruberg, L., Waksman, R., Satler, L. F., Pichard, A. D., and Kent, K. M. (2000). Novel approaches for the prevention of restenosis. Expert Opin Investig Drugs *9*, 2555-2578.

Gutierrez, P. S., Reis, M. M., Higuchi, M. L., Aiello, V. D., Stolf, N. A., and Lopes, E. A. (1998). Distribution of hyaluronan and dermatan/chondroitin sulfate proteoglycans in human aortic dissection. Connect Tissue Res *37*, 151-161.

Halayko, A. J., Salari, H., Ma, X., and Stephens, N. L. (1996). Markers of airway smooth muscle cell phenotype. Am J Physiol *270*, L1040-1051.

Halayko, A. J., and Solway, J. (2001). Molecular mechanisms of phenotypic plasticity in smooth muscle cells. J Appl Physiol *90*, 358-368.

Hamano, K., Ito, H., Fujimura, Y., Tsuboi, H., and Esato, K. (1998). Changes in the morphology and components of the coronary arteries during the progression of coronary arteriosclerosis following cardiac transplantation in rats. Cardiovasc Surg *6*, 296-301.

Han, C. I., Campbell, G. R., and Campbell, J. H. (2001). Circulating bone marrow cells can contribute to neointimal formation. J Vasc Res *38*, 113-119.

Hancock, W. W., Gao, W., Shemmeri, N., Shen, X. D., Gao, F., Busuttil, R. W., Zhai, Y., and Kupiec-Weglinski, J. W. (2002). Immunopathogenesis of accelerated allograft rejection in sensitized recipients: humoral and nonhumoral mechanisms. Transplantation *73*, 1392-1397.

Hancock, W. W., Shi, C., Picard, M. H., Bianchi, C., and Russell, M. E. (1995). LEW-to-F344 carotid artery allografts: analysis of a rat model of posttransplant vascular injury involving cell-mediated and humoral responses. Transplantation *60*, 1565-1572.

Hasenstab, D., Lea, H., Hart, C. E., Lok, S., and Clowes, A. W. (2000). Tissue factor overexpression in rat arterial neointima models thrombosis and progression of advanced atherosclerosis. Circulation *101*, 2651-2657.

Hassan, A. H., Lang, I. M., Ignatescu, M., Ullrich, R., Bonderman, D., Wexberg, P., Weidinger, F., and Glogar, H. D. (2001). Increased intimal apoptosis in coronary atherosclerotic vessel segments lacking compensatory enlargement. J Am Coll Cardiol *38*, 1333-1339.

Haunstetter, A., and Izumo, S. (1998). Apoptosis: basic mechanisms and implications for cardiovascular disease. Circ Res *82*, 1111-1129.

He, H., Stone, J. R., and Perkins, D. L. (2002). Analysis of robust innate immune response after transplantation in the absence of adaptive immunity. Transplantation *73*, 853-861.

Hedin, U., Bottger, B. A., Forsberg, E., Johansson, S., and Thyberg, J. (1988). Diverse effects of fibronectin and laminin on phenotypic properties of cultured arterial smooth muscle cells. J Cell Biol *107*, 307-319.

Hedin, U., Roy, J., Tran, P. K., Lundmark, K., and Rahman, A. (1999). Control of smooth muscle cell proliferation--the role of the basement membrane. Thromb Haemost *82 Suppl 1*, 23-26.

Heissig, B., Hattori, K., Dias, S., Friedrich, M., Ferris, B., Hackett, N. R., Crystal, R. G., Besmer, P., Lyden, D., Moore, M. A.*, et al.* (2002). Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell *109*, 625-637.

Hillebrands, J. L., Klatter, F. A., van den Hurk, B. M., Popa, E. R., Nieuwenhuis, P., and Rozing, J. (2001). Origin of neointimal endothelium and alpha-actin-positive smooth muscle cells in transplant arteriosclerosis. J Clin Invest *107*, 1411-1422.

Hirschi, K. K., and Goodell, M. A. (2002). Hematopoietic, vascular and cardiac fates of bone marrow-derived stem cells. Gene Ther *9*, 648-652.

Hixon, M. L., Obejero-Paz, C., Muro-Cacho, C., Wagner, M. W., Millie, E., Nagy, J., Hassold, T. J., and Gualberto, A. (2000). Cks1 mediates vascular smooth muscle cell polyploidization. J Biol Chem *275*, 40434-40442.

Holifield, B., Helgason, T., Jemelka, S., Taylor, A., Navran, S., Allen, J., and Seidel, C. (1996). Differentiated vascular myocytes: are they involved in neointimal formation? J Clin Invest *97*, 814-825.

Holzknecht, Z. E., Kuypers, K. L., Plummer, T. B., Williams, J., Bustos, M., Gores, G. J., Brunn, G. J., and Platt, J. L. (2002). Apoptosis and cellular activation in the pathogenesis of acute vascular rejection. Circ Res *91*, 1135-1141.

Hruban, R. H., Long, P. P., Perlman, E. J., Hutchins, G. M., Baumgartner, W. A., Baughman, K. L., and Griffin, C. A. (1993). Fluorescence in situ hybridization for the Y-

chromosome can be used to detect cells of recipient origin in allografted hearts following cardiac transplantation. Am J Pathol *142*, 975-980.

Hu, Y., Davison, F., Ludewig, B., Erdel, M., Mayr, M., Url, M., Dietrich, H., and Xu, Q. (2002a). Smooth muscle cells in transplant atherosclerotic lesions are originated from recipients, but not bone marrow progenitor cells. Circulation *106*, 1834-1839.

Hu, Y., Mayr, M., Metzler, B., Erdel, M., Davison, F., and Xu, Q. (2002b). Both donor and recipient origins of smooth muscle cells in vein graft atherosclerotic lesions. Circ Res *91*, e13-20.

Hultgardh-Nilsson, A., Lovdahl, C., Blomgren, K., Kallin, B., and Thyberg, J. (1997). Expression of phenotype- and proliferation-related genes in rat aortic smooth muscle cells in primary culture. Cardiovasc Res *34*, 418-430.

Ikari, Y., Yee, K. O., and Schwartz, S. M. (2000). Role of alpha5beta1 and alphavbeta3 integrins on smooth muscle cell spreading and migration in fibrin gels. Thromb Haemost *84*, 701-705.

Imanishi, T., Han, D. K., Hofstra, L., Hano, T., Nishio, I., Conrad Liles, W., Gorden, A. M., and Schwartz, S. M. (2002). Apoptosis of vascular smooth muscle cells is induced by Fas ligand derived from monocytes/macrophage. Atherosclerosis *161*, 143-151.

Izutani, H., Miyagawa, S., Shirakura, R., Matsumiya, G., Nakata, S., Shimazaki, Y., and Matsuda, H. (1995). Evidence that graft coronary arteriosclerosis begins in the early phase after transplantation and progresses without chronic immunoreaction. Histopathological analysis using a retransplantation model. Transplantation *60*, 1073-1079.

Janczewska, S., Ziolkowska, A., Durlik, M., Olszewski, W. L., and Lukomska, B. (1999). Fast lymphoid reconstitution after vascularized bone marrow transplantation in lethally irradiated rats. Transplantation *68*, 201-209.

Janczewska, S., Ziolkowska, A., Interewicz, B., Majewski, T., Olszewski, W. L., and Lukomska, B. (2000). Vascularized bone marrow transplanted in orthotopic hind-limb stimulates hematopoietic recovery in total-body-irradiated rats. Transpl Int *13 Suppl 1*, S541-546.

Jawien, A., Bowen-Pope, D. F., Lindner, V., Schwartz, S. M., and Clowes, A. W. (1992). Platelet-derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. J Clin Invest *89*, 507-511.

Johansson, C. B., Momma, S., Clarke, D. L., Risling, M., Lendahl, U., and Frisen, J. (1999). Identification of a neural stem cell in the adult mammalian central nervous system. Cell *96*, 25-34.

Kaiser, M., Weyand, C. M., Bjornsson, J., and Goronzy, J. J. (1998). Platelet-derived growth factor, intimal hyperplasia, and ischemic complications in giant cell arteritis. Arthritis Rheum *41*, 623-633.

Kataoka, T., Honda, Y., Bonneau, H. N., Yock, P. G., and Fitzgerald, P. J. (2002). New catheter-based technology for the treatment of restenosis. J Interv Cardiol *15*, 371-379.

Keck, B. M., Bennett, L. E., Fiol, B. S., Daily, O. P., Novick, R. J., and Hosenpud, J. D. (1997). Worldwide thoracic organ transplantation: a report from the UNOS/ISHLT International Registry for thoracic organ transplantation. Clin Transpl, 29-43.

Kiener, P. A., Davis, P. M., Rankin, B. M., Klebanoff, S. J., Ledbetter, J. A., Starling, G. C., and Liles, W. C. (1997a). Human monocytic cells contain high levels of intracellular Fas ligand: rapid release following cellular activation. J Immunol *159*, 1594-1598.

Kiener, P. A., Davis, P. M., Starling, G. C., Mehlin, C., Klebanoff, S. J., Ledbetter, J. A., and Liles, W. C. (1997b). Differential induction of apoptosis by Fas-Fas ligand interactions in human monocytes and macrophages. J Exp Med *185*, 1511-1516.

Korbling, M., Katz, R. L., Khanna, A., Ruifrok, A. C., Rondon, G., Albitar, M., Champlin, R. E., and Estrov, Z. (2002). Hepatocytes and epithelial cells of donor origin in recipients of peripheral-blood stem cells. N Engl J Med *346*, 738-746.

Kouwenhoven, E. A., JN, I. J., and de Bruin, R. W. (2000). Etiology and pathophysiology of chronic transplant dysfunction. Transpl Int *13*, 385-401.

Labarrere, C. A., Nelson, D. R., and Park, J. W. (2001). Pathologic markers of allograft arteriopathy: insight into the pathophysiology of cardiac allograft chronic rejection. Curr Opin Cardiol *16*, 110-117.

Landymore, R. W., MacAulay, M. A., and Manku, M. S. (1990). The effects of low, medium and high dose aspirin on intimal proliferation in autologous vein grafts used for arterial reconstruction. Eur J Cardiothorac Surg *4*, 441-444.

Landymore, R. W., Manku, M. S., Tan, M., MacAulay, M. A., and Sheridan, B. (1989). Effects of low-dose marine oils on intimal hyperplasia in autologous vein grafts. J Thorac Cardiovasc Surg *98*, 788-791.

Law, R. E., Meehan, W. P., Xi, X. P., Graf, K., Wuthrich, D. A., Coats, W., Faxon, D., and Hsueh, W. A. (1996). Troglitazone inhibits vascular smooth muscle cell growth and intimal hyperplasia. J Clin Invest *98*, 1897-1905.

Lee, R. S., Yamada, K., Houser, S. L., Womer, K. L., Maloney, M. E., Rose, H. S., Sayegh, M. H., and Madsen, J. C. (2001). Indirect recognition of allopeptides promotes the development of cardiac allograft vasculopathy. Proc Natl Acad Sci U S A *98*, 3276-3281.

Li, G., Chen, S. J., Oparil, S., Chen, Y. F., and Thompson, J. A. (2000). Direct in vivo evidence demonstrating neointimal migration of adventitial fibroblasts after balloon injury of rat carotid arteries. Circulation *101*, 1362-1365.

Li, J., Han, X., Jiang, J., Zhong, R., Williams, G. M., Pickering, J. G., and Chow, L. H. (2001). Vascular smooth muscle cells of recipient origin mediate intimal expansion after aortic allotransplantation in mice. Am J Pathol *158*, 1943-1947.

Libby, P. (2000). Coronary artery injury and the biology of atherosclerosis: inflammation, thrombosis, and stabilization. Am J Cardiol *86*, 3J-8J; discussion 8J-9J.

Libby, P. (2002). Inflammation in atherosclerosis. Nature *420*, 868-874.

Libby, P., and Pober, J. S. (2001). Chronic rejection. Immunity *14*, 387-397.

Lim, T. T., Liang, D. H., Botas, J., Schroeder, J. S., Oesterle, S. N., and Yeung, A. C. (1997). Role of compensatory enlargement and shrinkage in transplant coronary artery disease. Serial intravascular ultrasound study. Circulation *95*, 855-859.

Lindner, V., and Reidy, M. A. (1991). Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. Proc Natl Acad Sci U S A *88*, 3739-3743.

Liu, G., and Butany, J. (1992). Morphology of graft arteriosclerosis in cardiac transplant recipients. Hum Pathol *23*, 768-773.

Liu, Y., and Rao, M. S. (2003). Transdifferentiation-fact or artifact. J Cell Biochem *88*, 29-40.

Liu, Z., Sun, Y. K., Xi, Y. P., Maffei, A., Reed, E., Harris, P., and Suciu-Foca, N. (1993). Contribution of direct and indirect recognition pathways to T cell alloreactivity. J Exp Med *177*, 1643-1650.

Lu, C. Y. (1996). Ischemia, injury, and renal allograft rejection. Curr Opin Nephrol Hypertens *5*, 107-110.

Lukomska, B., Janczewska, S., Durlik, M., and Olszewski, W. L. (2000). Kinetics of bone marrow repopulation in lethally irradiated rats after transplantation of vascularized bone marrow in syngeneic hind limb. Ann Transplant *5*, 14-20.

Lupinetti, F. M., Tsai, T. T., and Kneebone, J. M. (1993). Endothelial cell replication in an in vivo model of aortic allografts. Ann Thorac Surg *56*, 237-241.

Luscher, T. F., and Tanner, F. C. (1993). Endothelial regulation of vascular tone and growth. Am J Hypertens *6*, 283S-293S.

Maalej, N., and Folts, J. D. (1996). Increased shear stress overcomes the antithrombotic platelet inhibitory effect of aspirin in stenosed dog coronary arteries. Circulation *93*, 1201-1205.

Mauiyyedi, S., and Colvin, R. B. (2002). Humoral rejection in kidney transplantation: new concepts in diagnosis and treatment. Curr Opin Nephrol Hypertens *11*, 609-618.

Mennander, A., Paavonen, T., and Hayry, P. (1993). Intimal thickening and medial necrosis in allograft arteriosclerosis (chronic rejection) are independently regulated. Arterioscler Thromb *13*, 1019-1025.

Mennander, A., Tiisala, S., Halttunen, J., Yilmaz, S., Paavonen, T., and Hayry, P. (1991). Chronic rejection in rat aortic allografts. An experimental model for transplant arteriosclerosis. Arterioscler Thromb *11*, 671-680.

Miller, L. W., Granville, D. J., Narula, J., and McManus, B. M. (2001). Apoptosis in cardiac transplant rejection. Cardiol Clin *19*, 141-154.

Mohacsi, P. J., Tuller, D., Hulliger, B., and Wijngaard, P. L. (1997). Different inhibitory effects of immunosuppressive drugs on human and rat aortic smooth muscle and endothelial cell proliferation stimulated by platelet-derived growth factor or endothelial cell growth factor. J Heart Lung Transplant *16*, 484-492.

Moliterno, D., and Topol, E. (1998). Restenosis: epidemiology and treatment. In Textbook of Cardiovascular Medicine, EJ Topol, (Philadelphia, Lippincott-Raven Publ) , pp2065-2100.

Mukherjee, D., Lingam, P., Chetcuti, S., Grossman, P. M., Moscucci, M., Luciano, A. E., and Eagle, K. A. (2002). Missed opportunities to treat atherosclerosis in patients undergoing peripheral vascular interventions: insights from the University of Michigan Peripheral Vascular Disease Quality Improvement Initiative (PVD-QI2). Circulation *106*, 1909-1912.

Mulukutla, S. R., and Cohen, H. A. (2002). Feasibility and efficacy of transradial access for coronary interventions in patients with acute myocardial infarction. Catheter Cardiovasc Interv *57*, 167-171.

Nagai, R., Suzuki, T., Aizawa, K., Miyamoto, S., Amaki, T., Kawai-Kowase, K., Sekiguchi, K. I., and Kurabayashi, M. (2001). Phenotypic modulation of vascular smooth muscle cells: dissection of transcriptional regulatory mechanisms. Ann N Y Acad Sci *947*, 56-66; discussion 66-57.

Nagano, H., Libby, P., Taylor, M. K., Hasegawa, S., Stinn, J. L., Becker, G., Tilney, N. L., and Mitchell, R. N. (1998). Coronary arteriosclerosis after T-cell-mediated injury in transplanted mouse hearts: role of interferon-gamma. Am J Pathol *152*, 1187-1197.

Neuville, P., Geinoz, A., Benzonana, G., Redard, M., Gabbiani, F., Ropraz, P., and Gabbiani, G. (1997). Cellular retinol-binding protein-1 is expressed by distinct subsets of rat arterial smooth muscle cells in vitro and in vivo. Am J Pathol *150*, 509-521.

Newby, A. C. (2000). An overview of the vascular response to injury: a tribute to the late Russell Ross. Toxicol Lett *112-113*, 519-529.

Nierhoff, D., Horvath, H. C., Mytilineos, J., Golling, M., Bud, O., Klar, E., Opelz, G., Voso, M. T., Ho, A. D., Haas, R., and Hohaus, S. (2000). Microchimerism in bone marrow-derived CD34(+) cells of patients after liver transplantation. Blood *96*, 763-767.

NIH (2002). Stem Cells: A Primer. National Institut of Health.

Oberman, A., Kuller, L. H., and Carleton, R. A. (1994). Prevention of cardiovascular disease--opportunities for progress. Prev Med *23*, 727-732.

Okimoto, T., Imazu, M., Hayashi, Y., Gomyo, Y., Sekiguchi, Y., Yanagihara, K., Shimohara, Y., Sumii, K., Yamamoto, H., Tadehara, F.*, et al.* (2001). Quinapril with high affinity to tissue angiotensin-converting enzyme reduces restenosis after percutaneous transcatheter coronary intervention. Cardiovasc Drugs Ther *15*, 323-329.

Olszewski, W. L., Durlik, M., Lukomska, B., Religa, P., Ziolkowska, H., Janczewska, S., Cybulska, E., Soin, J., Gaciong, Z., and Interewicz, B. (1999). DNA from rejecting allografts can be detected in recipient nonlymphoid tissues. Ann Transplant *4*, 39-41.

Olyaei, A. J., de Mattos, A. M., and Bennett, W. M. (2001). Nephrotoxicity of immunosuppressive drugs: new insight and preventive strategies. Curr Opin Crit Care *7*, 384-389.

Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S. M., Li, B., Pickel, J., McKay, R., Nadal-Ginard, B., Bodine, D. M.*, et al.* (2001). Bone marrow cells regenerate infarcted myocardium. Nature *410*, 701-705.

Orosz, C. G., and Pelletier, R. P. (1997). Chronic remodeling pathology in grafts. Curr Opin Immunol *9*, 676-680.

Pardue, M. L., and Gall, J. G. (1969). Molecular hybridization of radioactive DNA to the DNA of cytological preparations. Proc Natl Acad Sci U S A *64*, 600-604.

Pascual, G., Jurado, F., Rodriguez, M., Corrales, C., Lopez-Hervas, P., Bellon, J. M., and Bujan, J. (2002). The use of ischaemic vessels as prostheses or tissue engineering scaffolds after cryopreservation. Eur J Vasc Endovasc Surg *24*, 23-30.

Pasterkamp, G., de Kleijn, D. P., and Borst, C. (2000). Arterial remodeling in atherosclerosis, restenosis and after alteration of blood flow: potential mechanisms and clinical implications. Cardiovasc Res *45*, 843-852.

Paul, L. C. (1995). Chronic renal transplant loss. Kidney Int *47*, 1491-1499.

Petersen, B. E., Bowen, W. C., Patrene, K. D., Mars, W. M., Sullivan, A. K., Murase, N., Boggs, S. S., Greenberger, J. S., and Goff, J. P. (1999). Bone marrow as a potential source of hepatic oval cells. Science *284*, 1168-1170.

Pethig, K., Heublein, B., and Haverich, A. (2000). Vascular remodeling in cardiac allograft vasculopathy. Z Kardiol *89 Suppl 9*, IX/36-39.

Pinsky, D. J., Naka, Y., Liao, H., Oz, M. C., Wagner, D. D., Mayadas, T. N., Johnson, R. C., Hynes, R. O., Heath, M., Lawson, C. A., and Stern, D. M. (1996). Hypoxia-induced exocytosis of endothelial cell Weibel-Palade bodies. A mechanism for rapid neutrophil recruitment after cardiac preservation. J Clin Invest *97*, 493-500.

Pintucci, G., Steinberg, B. M., Seghezzi, G., Yun, J., Apazidis, A., Baumann, F. G., Grossi, E. A., Colvin, S. B., Mignatti, P., and Galloway, A. C. (1999). Mechanical endothelial damage results in basic fibroblast growth factor-mediated activation of extracellular signal-regulated kinases. Surgery *126*, 422-427.

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S., and Marshak, D. R. (1999). Multilineage potential of adult human mesenchymal stem cells. Science *284*, 143-147.

Plissonnier, D., Henaff, M., Poncet, P., Paris, E., Tron, F., Thuillez, C., and Michel, J. B. (2000). Involvement of antibody-dependent apoptosis in graft rejection. Transplantation *69*, 2601-2608.

Plissonnier, D., Nochy, D., Poncet, P., Mandet, C., Hinglais, N., Bariety, J., and Michel, J. B. (1995). Sequential immunological targeting of chronic experimental arterial allograft. Transplantation *60*, 414-424.

Powell, R. J., Cronenwett, J. L., Fillinger, M. F., Wagner, R. J., and Sampson, L. N. (1996). Endothelial cell modulation of smooth muscle cell morphology and organizational growth pattern. Ann Vasc Surg *10*, 4-10.

Priller, J., Persons, D. A., Klett, F. F., Kempermann, G., Kreutzberg, G. W., and Dirnagl, U. (2001). Neogenesis of cerebellar Purkinje neurons from gene-marked bone marrow cells in vivo. J Cell Biol *155*, 733-738.

Radovancevic, B., Poindexter, S., Birovljev, S., Velebit, V., McAllister, H. A., Duncan, J. M., Vega, D., Lonquist, J., Burnett, C. M., and Frazier, O. H. (1990). Risk factors for

development of accelerated coronary artery disease in cardiac transplant recipients. Eur J Cardiothorac Surg *4*, 309-312; discussion 313.

Rafii, S., Oz, M. C., Seldomridge, J. A., Ferris, B., Asch, A. S., Nachman, R. L., Shapiro, F., Rose, E. A., and Levin, H. R. (1995). Characterization of hematopoietic cells arising on the textured surface of left ventricular assist devices. Ann Thorac Surg *60*, 1627-1632.

Raisanen-Sokolowski, A., Vuoristo, P., Myllarniemi, M., Yilmaz, S., Kallio, E., and Hayry, P. (1995). Mycophenolate mofetil (MMF, RS-61443) inhibits inflammation and smooth muscle cell proliferation in rat aortic allografts. Transpl Immunol *3*, 342-351.

Rebocho, M. J., Aguiar, C., and Queiroz e Melo, J. (2001). [Morbidity and mortality after heart transplanation]. Rev Port Cardiol *20 Suppl 3*, 67-74.

Regar, E., Serruys, P. W., Bode, C., Holubarsch, C., Guermonprez, J. L., Wijns, W., Bartorelli, A., Constantini, C., Degertekin, M., Tanabe, K*., et al.* (2002). Angiographic findings of the multicenter Randomized Study With the Sirolimus-Eluting Bx Velocity Balloon-Expandable Stent (RAVEL): sirolimus-eluting stents inhibit restenosis irrespective of the vessel size. Circulation *106*, 1949-1956.

Reidy, M. A., and Lindner, V. (1991). Basic FGF and growth of arterial cells. Ann N Y Acad Sci *638*, 290-299.

Rose, M. L. (1998). Endothelial cells as antigen-presenting cells: role in human transplant rejection. Cell Mol Life Sci *54*, 965-978.

Ross, M. H., Romrell, L. J., and Kaye, G. I. (1995). Histology a text and atlas. Muscle tissue, Cardiovascular System *third edition*, 214-255, 302-329.

Ross, R. (1999). Atherosclerosis is an inflammatory disease. Am Heart J *138*, S419-420.

Rossmann, P., and Lacha, J. (1999). Ultrastructure of rat aortic grafts. Folia Microbiol (Praha) *44*, 567-578.

Rossmann, P., Lacha, J., and Lodererova, A. (1999). Morphology and immunohistochemistry of rat aortic grafts. Folia Microbiol (Praha) *44*, 339-353.

Roy, J. (2001). Extracellular Matrix-Mediated Signaling in the Regulation of Vascular SMC Phenotype and Function. Diss. Med., Karolinska Institutet, 2001.

Roy, J., Tran, P. K., Religa, P., Kazi, M., Henderson, B., Lundmark, K., and Hedin, U. (2002). Fibronectin promotes cell cycle entry in smooth muscle cells in primary culture. Exp Cell Res *273*, 169-177.

Saleem, S., Konieczny, B. T., Lowry, R. P., Baddoura, F. K., and Lakkis, F. G. (1996). Acute rejection of vascularized heart allografts in the absence of IFNgamma. Transplantation *62*, 1908-1911.

Sallam, M., Spanos, V., Briguori, C., Di Mario, C., Tzifos, V., Dharmadhikari, A., Albiero, R., and Colombo, A. (2001). Predictors of re-occlusion after successful recanalization of chronic total occlusion. J Invasive Cardiol *13*, 511-515.

Salom, R. N., Maguire, J. A., Esmore, D., and Hancock, W. W. (1994). Analysis of proliferating cell nuclear antigen expression aids histological diagnosis and is predictive of progression of human cardiac allograft rejection. Am J Pathol *145*, 876-882.

Sata, M., Saiura, A., Kunisato, A., Tojo, A., Okada, S., Tokuhisa, T., Hirai, H., Makuuchi, M., Hirata, Y., and Nagai, R. (2002). Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. Nat Med *8*, 403-409.

Schmid, C., Heemann, U., and Tilney, N. L. (1996). Retransplantation reverses mononuclear infiltration but not myointimal proliferation in a rat model of chronic cardiac allograft rejection. Transplantation *61*, 1695-1699.

Schmitz-Rixen, T., Megerman, J., Colvin, R. B., Williams, A. M., and Abbott, W. M. (1988). Immunosuppressive treatment of aortic allografts. J Vasc Surg *7*, 82-92.

Schneeberger, H., Schleibner, S., Illner, W. D., Messmer, K., and Land, W. (1993). The impact of free radical-mediated reperfusion injury on acute and chronic rejection events following cadaveric renal transplantation. Clin Transpl, 219-232.

Schwartz, L., and Seidelin, P. H. (1995). Antithrombotic and thrombolytic therapy in patients undergoing coronary artery interventions: a review. Prog Cardiovasc Dis *38*, 67-86.

Schwartz, R. S., and Henry, T. D. (2002). Pathophysiology of coronary artery restenosis. Rev Cardiovasc Med *3 Suppl 5*, S4-9.

Schwartz, S. M., deBlois, D., and O'Brien, E. R. (1995). The intima. Soil for atherosclerosis and restenosis. Circ Res *77*, 445-465.

Schwartz, S. M., Stemerman, M. B., and Benditt, E. P. (1975). The aortic intima. II. Repair of the aortic lining after mechanical denudation. Am J Pathol *81*, 15-42.

Schwartz, S. M., Virmani, R., and Rosenfeld, M. E. (2000). The good smooth muscle cells in atherosclerosis. Curr Atheroscler Rep *2*, 422-429.

Schwarzacher, S. P., Uren, N. G., Ward, M. R., Schwarzkopf, A., Giannetti, N., Hunt, S., Fitzgerald, P. J., Oesterle, S. N., and Yeung, A. C. (2000). Determinants of coronary remodeling in transplant coronary disease: a simultaneous intravascular ultrasound and Doppler flow study. Circulation *101*, 1384-1389.

Sharabi, Y., Grotto, I., Huerta, M., Eldad, A., and Green, M. S. (2001). Risk factor profile for atherosclerosis among young adults in Israel--results of a large-scale survey from the young adult periodic examinations in Israel (YAPEIS) database. Eur J Epidemiol *17*, 757-764.

Shi, Y., O'Brien, J. E., Jr., Fard, A., and Zalewski, A. (1996). Transforming growth factor-beta 1 expression and myofibroblast formation during arterial repair. Arterioscler Thromb Vasc Biol *16*, 1298-1305.

Shi, Y., Patel, S., Niculescu, R., Chung, W., Desrochers, P., and Zalewski, A. (1999). Role of matrix metalloproteinases and their tissue inhibitors in the regulation of coronary cell migration. Arterioscler Thromb Vasc Biol *19*, 1150-1155.

Shimizu, K., Sugiyama, S., Aikawa, M., Fukumoto, Y., Rabkin, E., Libby, P., and Mitchell, R. N. (2001). Host bone-marrow cells are a source of donor intimal smooth- muscle-like cells in murine aortic transplant arteriopathy. Nat Med *7*, 738-741.

Simper, D., Stalboerger, P. G., Panetta, C. J., Wang, S., and Caplice, N. M. (2002). Smooth muscle progenitor cells in human blood. Circulation *106*, 1199-1204.

Sjolund, M., Rahm, M., Claesson-Welsh, L., Sejersen, T., Heldin, C. H., and Thyberg, J. (1990). Expression of PDGF alpha- and beta-receptors in rat arterial smooth muscle cells is phenotype and growth state dependent. Growth Factors *3*, 191-203.

Skoog, T., Dichtl, W., Boquist, S., Skoglund-Andersson, C., Karpe, F., Tang, R., Bond, M. G., de Faire, U., Nilsson, J., Eriksson, P., and Hamsten, A. (2002). Plasma tumour necrosis factor-alpha and early carotid atherosclerosis in healthy middle-aged men. Eur Heart J *23*, 376-383.

Sobue, K., Hayashi, K., and Nishida, W. (1999). Expressional regulation of smooth muscle cell-specific genes in association with phenotypic modulation. Mol Cell Biochem *190*, 105-118.

Soderberg-Naucler, C., and Emery, V. C. (2001). Viral infections and their impact on chronic renal allograft dysfunction. Transplantation *71*, SS24-30.

Southgate, K. M., Fisher, M., Banning, A. P., Thurston, V. J., Baker, A. H., Fabunmi, R. P., Groves, P. H., Davies, M., and Newby, A. C. (1996). Upregulation of basement membrane-degrading metalloproteinase secretion after balloon injury of pig carotid arteries. Circ Res *79*, 1177-1187.

Spaet, T. H., Stemerman, M. B., Veith, F. J., and Lejnieks, I. (1975). Intimal injury and regrowth in the rabbit aorta; medial smooth muscle cells as a source of neointima. Circ Res *36*, 58-70.

Stamm, C., Westphal, B., Kleine, H. D., Petzsch, M., Kittner, C., Klinge, H., Schumichen, C., Nienaber, C. A., Freund, M., and Steinhoff, G. (2003). Autologous bone-marrow stem-cell transplantation for myocardial regeneration. Lancet *361*, 45-46.

Stary, H. C., Chandler, A. B., Dinsmore, R. E., Fuster, V., Glagov, S., Insull, W., Jr., Rosenfeld, M. E., Schwartz, C. J., Wagner, W. D., and Wissler, R. W. (1995). A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation *92*, 1355-1374.

Stoica, S. C., Goddard, M., and Large, S. R. (2002). The endothelium in clinical cardiac transplantation. Ann Thorac Surg *73*, 1002-1008.

Takada, M., Nadeau, K. C., Hancock, W. W., Mackenzie, H. S., Shaw, G. D., Waaga, A. M., Chandraker, A., Sayegh, M. H., and Tilney, N. L. (1998). Effects of explosive brain death on cytokine activation of peripheral organs in the rat. Transplantation *65*, 1533-1542.

Tashiro, H., Fukuda, Y., Kimura, A., Hoshino, S., Ito, H., and Dohi, K. (1996). Assessment of microchimerism in rat liver transplantation by polymerase chain reaction. Hepatology *23*, 828-834.

Tejani, A., and Emmett, L. (2001). Acute and chronic rejection. Semin Nephrol *21*, 498-507.

Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D. M., Nakano, Y., Meyer, E. M., Morel, L., Petersen, B. E., and Scott, E. W. (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature *416*, 542-545.

Thyberg, J. (1996). Differentiated properties and proliferation of arterial smooth muscle cells in culture. Int Rev Cytol *169*, 183-265.

Thyberg, J. (1998). Phenotypic modulation of smooth muscle cells during formation of neointimal thickenings following vascular injury. Histol Histopathol *13*, 871-891.

Thyberg, J., Blomgren, K., Hedin, U., and Dryjski, M. (1995). Phenotypic modulation of smooth muscle cells during the formation of neointimal thickenings in the rat carotid artery after balloon injury: an electron-microscopic and stereological study. Cell Tissue Res *281*, 421-433.

Thyberg, J., Blomgren, K., Roy, J., Tran, P. K., and Hedin, U. (1997). Phenotypic modulation of smooth muscle cells after arterial injury is associated with changes in the distribution of laminin and fibronectin. J Histochem Cytochem *45*, 837-846.

Thyberg, J., Palmberg, L., Nilsson, J., Ksiazek, T., and Sjolund, M. (1983). Phenotype modulation in primary cultures of arterial smooth muscle cells. On the role of platelet-derived growth factor. Differentiation *25*, 156-167.

Tullius, S. G., and Tilney, N. L. (1995). Both alloantigen-dependent and -independent factors influence chronic allograft rejection. Transplantation *59*, 313-318.

Urist, M. R., and Adams, J. M., Jr. (1967). Localization mechanism of calcification in transplants of aorta. Ann Surg *166*, 1-18.

Waaga, A. M., Gasser, M., Laskowski, I., and Tilney, N. L. (2000). Mechanisms of chronic rejection. Curr Opin Immunol *12*, 517-521.

Wakitani, S., Goto, T., Pineda, S. J., Young, R. G., Mansour, J. M., Caplan, A. I., and Goldberg, V. M. (1994). Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. J Bone Joint Surg Am *76*, 579-592.

Waller, B. F., Rothbaum, D. A., Gorfinkel, H. J., Ulbright, T. M., Linnemeier, T. J., and Berger, S. M. (1984). Morphologic observations after percutaneous transluminal balloon angioplasty of early and late aortocoronary saphenous vein bypass grafts. J Am Coll Cardiol *4*, 784-792.

Waltenberger, J., Akyurek, M. L., Aurivillius, M., Wanders, A., Larsson, E., Fellstrom, B., and Funa, K. (1996). Ischemia-induced transplant arteriosclerosis in the rat. Induction of peptide growth factor expression. Arterioscler Thromb Vasc Biol *16*, 1516-1523.

Walter, D. H., Rittig, K., Bahlmann, F. H., Kirchmair, R., Silver, M., Murayama, T., Nishimura, H., Losordo, D. W., Asahara, T., and Isner, J. M. (2002). Statin therapy accelerates reendothelialization: a novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. Circulation *105*, 3017-3024.

Van Damme, A., Vanden Driessche, T., Collen, D., and Chuah, M. K. (2002). Bone marrow stromal cells as targets for gene therapy. Curr Gene Ther *2*, 195-209.

VanBuskirk, A. M., Pidwell, D. J., Adams, P. W., and Orosz, C. G. (1997). Transplantation immunology. Jama *278*, 1993-1999.

Watson, K. E., and Demer, L. L. (1996). The atherosclerosis-calcification link? Curr Opin Lipidol *7*, 101-104.

Wei, G. L., Krasinski, K., Kearney, M., Isner, J. M., Walsh, K., and Andres, V. (1997). Temporally and spatially coordinated expression of cell cycle regulatory factors after angioplasty. Circ Res *80*, 418-426.

Verfaillie, C. M. (2002). Adult stem cells: assessing the case for pluripotency. Trends Cell Biol *12*, 502-508.

Wilkinson, A. (2001). Progress in the clinical application of immunosuppressive drugs in renal transplantation. Curr Opin Nephrol Hypertens *10*, 763-770.

Williams, G. M., and Alvarez, C. A. (1969). Host repopulation of the endothelium in allografts of kidneys and aorta. Surg Forum *20*, 293-294.

Virmani, R., Liistro, F., Stankovic, G., Di Mario, C., Montorfano, M., Farb, A., Kolodgie, F. D., and Colombo, A. (2002). Mechanism of late in-stent restenosis after implantation of a paclitaxel derivate-eluting polymer stent system in humans. Circulation *106*, 2649-2651.

Womer, K. L., Vella, J. P., and Sayegh, M. H. (2000). Chronic allograft dysfunction: mechanisms and new approaches to therapy. Semin Nephrol *20*, 126-147.

Wood, K. J., Hopley, A., Dallman, M. J., and Morris, P. J. (1988). Lack of correlation between the induction of donor class I and class II major histocompatibility complex antigens and graft rejection. Transplantation *45*, 759-767.

Yamashita, J., Itoh, H., Hirashima, M., Ogawa, M., Nishikawa, S., Yurugi, T., Naito, M., and Nakao, K. (2000). Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. Nature *408*, 92-96.

Yankah, A. C., and Wottge, H. U. (1997). Allograft conduit wall calcification in a model of chronic arterial graft rejection. J Card Surg *12*, 86-92.

Yee, K. O., Rooney, M. M., Giachelli, C. M., Lord, S. T., and Schwartz, S. M. (1998). Role of beta1 and beta3 integrins in human smooth muscle cell adhesion to and contraction of fibrin clots in vitro. Circ Res *83*, 241-251.

Ying, Q. L., Nichols, J., Evans, E. P., and Smith, A. G. (2002). Changing potency by spontaneous fusion. Nature *416*, 545-548.

Young, J. B. (2000). Perspectives on cardiac allograft vasculopathy. Curr Atheroscler Rep *2*, 259-271.

Yutani, C., Imakita, M., Ishibashi-Ueda, H., Tsukamoto, Y., Nishida, N., and Ikeda, Y. (1999). Coronary atherosclerosis and interventions: pathological sequences and restenosis. Pathol Int *49*, 273-290.

Zalewski, A., and Shi, Y. (1997). Vascular myofibroblasts. Lessons from coronary repair and remodeling. Arterioscler Thromb Vasc Biol *17*, 417-422.

Zavazava, N., and Kabelitz, D. (2000). Alloreactivity and apoptosis in graft rejection and transplantation tolerance. J Leukoc Biol *68*, 167-174.