VASCULAR GROWTH FACTORS AND PROGENITOR CELLS IN CARDIAC ALLOGRAFT ARTERIOSCLEROSIS

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Academic Dissertation

To be publicly discussed with the permission of the Medical Faculty, University of Helsinki, in small auditorium, Haartman Institute, on Friday, January 26th, 2007, at 12 o’clock noon

Helsinki 2007
To Sanna, Minja, Kasper and Marius
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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals:


* Equal contribution.
ABBREVIATIONS

AAV  adeno-associated virus
Ad  adenovirus
Ag  antigen
AMR  acute antibody-mediated rejection
Ang  angiopoietin
APC  antigen-presenting cell
α-SMA  α-smooth muscle actin
BM  bone marrow
CAD  coronary artery disease
CMV  cytomegalovirus
CsA  cyclosporine A
CSC  cardiac stem cell
EC  endothelial cell
ECM  extracellular matrix
EGF  epidermal growth factor
Egr-1  early growth response factor-1
eNOS  endothelial nitric oxide synthase
EPC  endothelial progenitor cell
FGF  fibroblast growth factor
G-CSF  granulocyte colony-stimulating factor
GFP  green fluorescent protein
GM-CSF  granulocyte-monocyte colony-stimulating factor
HB-EGF  heparin-binding EGF-like growth factor
HEV  high endothelial venule
HGF  hepatocyte growth factor
HIF  hypoxia-inducible factor
HLA  human leukocyte antigen
HRE  hypoxia response element
HSC  hematopoietic stem cell
ICAM-1  intercellular adhesion molecule-1
IFN-γ  interferon-γ
IL  interleukin
IP-10  interferon-inducible protein-10
IVUS  intravascular ultrasound
Luc  luciferase
MCP-1  monocyte chemoattractant protein-1
MHC  major histocompatibility complex
MMF  mycophenolate mofetil
MMP  matrix metalloproteinase
mTOR  mammalian target of rapamycin
NF-AT  nuclear factor of activated T cells
NK cell  natural killer cell
NO  nitric oxide
PDGF  platelet-derived growth factor
PDGFR  platelet-derived growth factor receptor
PI3K  phosphatidylinositol 3´-kinase
PIGF  placental growth factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>SDF-1</td>
<td>stromal cell derived factor-1</td>
</tr>
<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
</tr>
<tr>
<td>SP</td>
<td>side population</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>T&lt;sub&gt;reg&lt;/sub&gt;</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>TxCAD</td>
<td>cardiac allograft arteriosclerosis i.e. transplant coronary artery disease</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>vascular endothelial receptor</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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</table>
ABSTRACT

Heart transplantation is the only therapeutic modality for many end-stage heart diseases. Although the short-term results have significantly improved, poor long-term survival of heart transplant patients remains a challenging problem. The decline in long-term survival is mainly due to the development of cardiac allograft arteriosclerosis, i.e. transplant coronary artery disease (TxCAD) that is an accelerated form of coronary artery disease. Both traditional cardiovascular and transplantation-related immunological and non-immunological risk factors for TxCAD have been identified but options for therapy are limited.

TxCAD is considered as a poorly regulated healing process of the transplanted heart that involves dysfunction of cardiac allograft vascular cells – endothelial cells (EC) and smooth muscle cells (SMC). Activated EC in cardiac allografts function as antigen-presenting cells, regulate inflammatory cell entry and secrete soluble SMC growth factors. In turn, SMC and their progenitors invade the intima of the injured vessels and ultimately occlude the affected coronary arteries.

Different vascular growth factors have to be delicately regulated in vascular development. Adult vasculature is normally in a quiescent state, but ischemia-reperfusion injury and alloimmune inflammation may activate vascular cells in transplanted hearts. In the present study, experimental heterotopic transplantation models and in vitro experiments were used to study the role of vascular growth factors in the development of TxCAD. Specifically, the role of angiogenic and pro-inflammatory vascular endothelial growth factor (VEGF), EC growth factor angiopoietin (Ang), and SMC mitogen platelet-derived growth factor (PDGF) were investigated. Pharmacological and gene transfer approaches were used to target these vascular growth factors and to assess their therapeutic potential.

This study shows that alloimmune response in heart transplants upregulates expression of VEGF mainly in allograft-infiltrating macrophages and induces an angiogenic response involving primitive EC that mainly originate from donor cells. VEGF gene transfer to cardiac allografts with intracoronary adenoviral vector increased macrophage infiltration, intimal angiogenesis and TxCAD. Pharmacological VEGF inhibition with receptor tyrosine kinase inhibitor PTK787 decreased allograft inflammation and TxCAD, and simultaneous PDGF inhibition with imatinib further decreased TxCAD. Selective inhibition of two VEGF-receptors (VEGFR) with neutralizing antibodies markedly decreased allograft inflammation and TxCAD, and VEGFR-2 inhibition normalized the density of primitive and mature capillaries in the allografts. These results suggest novel therapeutic applications for anti-VEGF strategies to inhibit allograft inflammation, angiogenesis and development of TxCAD, and that additional PDGF inhibition may further decrease pathological SMC proliferation.

In contrast to VEGF, adenovirus-mediated transient Ang1 expression in the allograft had anti-inflammatory and anti-arteriosclerotic effects. Adeno-associated virus (AAV)-mediated prolonged Ang1 or Ang2 expression had similar anti-inflammatory effects. However, prolonged exposure to exogenous Ang1 activated SMC in allograft arteries whereas AAV-mediated Ang2 expression had no effects on SMC activation and decreased the development of TxCAD. These results show that angiopoietins have potent anti-inflammatory and anti-apoptotic effects in cardiac allografts but the timing of angiopoietin therapy has to be considered as prolonged Ang1 exposure may induce SMC activation. Importantly, AAV-vectors were identified as a
potential tool to introduce long-lasting expression of therapeutic genes in cardiac allografts.

To summarize, these studies indicate an interplay of inflammation, angiogenesis and arteriosclerosis in cardiac allografts, and show that vascular growth factors are important regulators in the process. The results suggest that VEGF inhibition, PDGF inhibition and angiopoietin therapy with clinically relevant pharmacological agents or novel gene therapy approaches may counteract vascular dysfunction in cardiac allografts, and have beneficial effects on the survival of heart transplant patients in the future.
INTRODUCTION

The first human heart transplantation was performed in 1967 (Barnard 1967). Still today, heart transplantation is the only available therapeutic option for many end-stage heart diseases and over 4000 heart transplantations are performed annually worldwide. Although the 1-year survival of heart transplant patients has gradually improved, almost 4% of patients are lost annually thereafter (Taylor et al. 2006). The poor long term survival is largely attributable to the development of cardiac allograft arteriosclerosis, i.e. transplant coronary artery disease (TxCAD) which is detected by multi-vessel intravascular ultrasound in 58% of recipients at 1 year and in 74% of recipients at 3 years after the heart transplantation (Kapadia et al. 2000).

TxCAD is characterized by concentric intimal thickening of the whole arterial network of the allograft – including large conducting coronaries and their smaller cardiomyocyte-perfusing branches (Arbustini and Roberts 1996). The pathogenesis of the disease is not completely understood but continuous immunological and non-immunological damage to the allograft arteries and endothelial cells (EC) is believed to trigger an uncontrolled repair process where smooth muscle cells (SMC) accumulate to the intima of the affected vessels (Libby and Pober 2001). The intimal SMC are traditionally believed to migrate from the media layer (Hayry et al. 1993) of the affected artery but recent experimental data imply that recipient-derived stem and progenitor cells may also contribute to the intimal growth (Saiura et al. 2001).

Currently, clinical tools to prevent TxCAD are limited and the only option to treat severe established disease is re-transplantation (Valantine 2004). The purpose of this study was to investigate the role of vascular growth factors and vascular progenitor cells in the pathogenesis of TxCAD using experimental heart transplantation models and in vitro experiments. Moreover, novel pharmacological and gene therapy approaches were used to identify potential therapeutic targets to inhibit the development of the disease.
REVIEW OF THE LITERATURE

1. Clinical heart transplantation

1.1. Introduction to clinical heart transplantation

The idea of replacing damaged organs has intrigued mankind for generations. Before heart transplantation was recognized as a standard clinical practice, extensive advances in surgical and anaesthesiological techniques, immunobiology and pharmacology, and the definition of brain death were required (Patterson and Patterson 1997). The first clinical heart transplantation was preceded with work on vascular anastomosis by Alexis Carrel in the beginning of the 20th century (Carrel and Guthrie 1905), and studies with experimental heart transplantation by Mann in 1930s (Mann et al. 1933) and Demikhov in 1950s (Demikhov 1962). In addition, development of clinical heart transplantation techniques in Stanford by Shumway and Lower were essential (Hurley et al. 1962). The first clinical heart transplantation was performed by Christiaan Barnard in Cape Town in 1967 (Barnard 1967). Although the patient died of pneumonia 18 days after the transplantation, many heart transplantation programs were initiated worldwide. The initial enthusiasm soon changed as most of the patients died only months after the operation because of acute rejection or infections. The invention (Borel et al. 1976) and introduction of a new immunosuppressive drug, cyclosporine A (CsA), in 1981 significantly improved the survival rates of heart transplant patients and made the procedure clinically relevant (Griffith et al. 1982). In the beginning of the 21st century, heart transplantation is the only therapeutic option for many end-stage heart diseases and about 4000 heart transplantations are performed annually worldwide (Taylor et al. 2006).

1.2. Indications and patient characteristics

Coronary heart failure (2%) and non-coronary cardiomyopathy (6%) are the two main indications for heart transplantation. In addition, heart transplantations are performed due to valvular (3%) or congenital causes (2%), or the need for re-transplantation (2%). Heart transplant recipients are relatively young although the mean age has increased to about 51 years. According to international registry information, about half of the heart transplant recipients are hospitalized and are on intravenous inotropes at the time of transplantation, and about 23% have left ventricular assist device. The average donor age is about 30 years and the cause of death is most commonly head trauma (66%) or stroke (23%) (Taylor et al. 2006).

1.3. Outcomes

Successful heart transplantation returns critically ill patients back to normal life (Mandak et al. 1995). The prognosis of medium and high risk patients is better compared to current optimal medical therapy (Lim et al. 2005). The patient survival after adult heart transplantation during different era is shown in Figure 1. Currently, adult heart transplant patient survival is about 90% at 6 months after the transplantation, and these early results have improved substantially over time. After the first year, the survival declines at a constant rate with almost 4% of patients lost per year. In contrast to the early results, survival after the first year has not improved much over time. Today, about half of the heart transplant patients are estimated to survive for more than 10 years (Taylor et al. 2005).
1.4. Complications and comorbidity

The complications and comorbidity after heart transplantation are mainly related to the transplantation procedure, alloimmune responses, pathological reparative responses, and side-effects of immunosuppressive drugs. Table 1 summarizes the causes of death at different time points after heart transplantation. During the first month and year after transplantation, the primary causes of death are graft failure, acute rejection and infections. After the first post-operative year, TxCAD and late graft failure - that is also likely due to TxCAD - account for more than 30% of the deaths combined (Taylor et al. 2005). Development of TxCAD also strongly predicts subsequent adverse events (Kobashigawa et al. 2005; Tuzcu et al. 2005). The proportion of malignancies, infections and renal failure increases with time, and these are considered complications of sustained immunosuppression. In contrast, the proportion of acute rejections decreases with time (Taylor et al. 2005). Lymphomas and skin cancer are the most common malignancies among heart transplant patients (Buell et al. 2005). Immunosuppression is also associated with metabolic problems. Within 1 year after heart transplantation, the cumulative prevalence of hypertension is 77%, hyperlipidemia 67%, and diabetes 30% (Taylor et al. 2005).

2. Immunobiology of heart transplantation

2.1. Introduction to the immunobiology of heart transplantation

A functional immune system is essential to defend off pathogens. Organ transplantation poses a challenge to the host immune system and the subsequent alloimmune reaction may be detrimental both to the transplant and to the recipient. The basic mechanisms underlying recognition of transplanted tissue as foreign started to unfold as Sir Peter Medawar studied why skin grafted to British Second World War fighter pilots did not survive (Medawar 1944).
Table 1. Cause of death after heart transplantation

<table>
<thead>
<tr>
<th>Cause of death</th>
<th>0-30 days</th>
<th>31 days-1 year</th>
<th>&gt;1-3 years</th>
<th>&gt;3-5 years</th>
<th>&gt;5 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>TxCAD</td>
<td>1.6 %</td>
<td>4.6 %</td>
<td>14.3 %</td>
<td>16.9 %</td>
<td>14.9 %</td>
</tr>
<tr>
<td>Graft failure</td>
<td>40.2 %</td>
<td>7.9 %</td>
<td>23.2 %</td>
<td>18.7 %</td>
<td>18.2 %</td>
</tr>
<tr>
<td>Malignancy</td>
<td>0.2 %</td>
<td>4.0 %</td>
<td>14.6 %</td>
<td>23.6 %</td>
<td>22.9 %</td>
</tr>
<tr>
<td>Non-CMV infection</td>
<td>12.9 %</td>
<td>32.7 %</td>
<td>13.3 %</td>
<td>9.4 %</td>
<td>10.0 %</td>
</tr>
<tr>
<td>Acute rejection</td>
<td>67.0 %</td>
<td>12.1 %</td>
<td>9.6 %</td>
<td>4.1 %</td>
<td>1.3 %</td>
</tr>
<tr>
<td>Technical</td>
<td>7.4 %</td>
<td>1.1 %</td>
<td>0.8 %</td>
<td>1.0 %</td>
<td>0.9 %</td>
</tr>
<tr>
<td>CMV</td>
<td>0.1 %</td>
<td>1.2 %</td>
<td>0.8 %</td>
<td>0.2 %</td>
<td>0.1 %</td>
</tr>
<tr>
<td>Multiorgan failure</td>
<td>14.0 %</td>
<td>10.1 %</td>
<td>5.1 %</td>
<td>5.8 %</td>
<td>8.2 %</td>
</tr>
<tr>
<td>Renal failure</td>
<td>0.6 %</td>
<td>0.8 %</td>
<td>1.6 %</td>
<td>3.6 %</td>
<td>6.0 %</td>
</tr>
<tr>
<td>Pulmonary</td>
<td>4.1 %</td>
<td>3.4 %</td>
<td>4.5 %</td>
<td>3.8 %</td>
<td>3.9 %</td>
</tr>
<tr>
<td>Cerebrovascular</td>
<td>6.4 %</td>
<td>4.1 %</td>
<td>3.2 %</td>
<td>3.3 %</td>
<td>4.1 %</td>
</tr>
<tr>
<td>Other</td>
<td>5.9 %</td>
<td>7.9 %</td>
<td>9.1 %</td>
<td>9.7 %</td>
<td>9.4 %</td>
</tr>
</tbody>
</table>

Data modified from the ISHLT registry report 2005 (Taylor et al. 2005). CMV, cytomegalovirus; TxCAD, cardiac allograft arteriosclerosis.

The key steps in the development of alloimmune response include activation of antigen-presenting cells (APC), recognition of the transplanted organ as foreign (allorecognition), activation and clonal expansion of alloreactive lymphocytes, and destruction of the allograft through cell-mediated and humoral mechanisms (effector phase) (Le Moine et al. 2002; Whitelegg and Barber 2004). Hyperacute rejection occurs in minutes after the heart transplantation due to preformed anti-donor antibodies and leads to rapid destruction of the allograft. Acute rejection may develop at any stage but is most common during the first 3 to 6 months after transplantation. It develops in days and involves T cell-dependent reactivity and/or alloantibody reactivity to donor antigens. The recently-revised classification of acute rejection is shown in Table 2. Chronic rejection can be viewed as a response to injury as discussed in detail in Chapter 3. It occurs within months to years after transplantation and involves chronic inflammation, fibrosis, cardiomyopathy and TxCAD (Hayry et al. 1993; Libby and Pober 2001).

### 2.2. Antigen-presenting cell activation

Activation of innate immunity and antigen-presenting cells (APC) such as dendritic cells and macrophages is a proximal step in the development of alloimmune responses (Figure 2). Although the adaptive immunity determines self and non-self, it is the tissue injury and the following APC activation that may actually determine the form of alloimmune response after transplantation (Matzinger 2002; Walker et al. 2006). The pattern recognition receptors – such as toll-like receptors (TLR) – form a molecular link between tissue injury, and the activation of innate and subsequent adaptive immunity. These receptors recognize exogenous signals such as bacterial lipopolysaccharide (Yang et al. 1998). In the context of organ transplantation, several endogenous signals related to tissue injury such as heat shock proteins (Schimke et al. 2000) and hyaluronan (Johnsson and Tufveson 2006) may also activate TLR and innate immunity (Andrade et al. 2005). Several studies show that activation of TLR and innate immunity plays a central role in alloimmune responses. The presence of hyporesponsive TLR4 polymorphism in lung transplant patients is associated with a decreased rate of acute rejections (Palmer et al. 2003). Also, experimental studies show that TLR signalling has a critical role in alloimmune activation and suppression of regulatory T cells (Goldstein et al. 2003; Chen et al. 2006; Walker et al. 2006). These results may in part explain why donor brain death provokes host immune...
system, accelerates acute rejection (Wilhelm et al. 2000) and impairs cardiac allograft survival (Karamlou et al. 2005).

Figure 2. Antigen-presenting cell (APC) activation in transplantation. APC may be activated through toll-like receptors (TLR) that recognize bacterial structures. In transplantation, TLR may recognize several endogenous molecules released by tissue injury related to donor brain death, ischemia-reperfusion injury, acute rejections or infections. Activated APC process transplant antigens, and upregulate expression of MHC, costimulatory molecules and chemokine receptors, and finally migrate to lymphoid organs for antigen presentation. Ag, antigen; APC, antigen-presenting cell; B7, costimulatory molecule (CD80 or 86); CCR7, chemokine receptor; HSP, heat shock protein; LPS, lipopolysaccharide; MHC, major histocompatibility factor; TLR, toll-like receptor.

2.3. Allorecognition

During allorecognition, transplant antigens are presented by APC to host T lymphocytes. In this process, the T cell receptor (TCR) on the surface of a lymphocyte interacts with the transplant antigen presented by the major histocompatibility complex molecule (MHC). If that specific TCR detects the transplant antigen - and proper co-stimulatory signals are present - the lymphocyte is activated and clonal expansion of the alloreactive lymphocyte population occurs (Whitelegg and Barber 2004; Male et al. 2006). Antigen presentation occurs in regional lymph nodes after skin transplantation (Lakkis et al. 2000) whereas both lymph nodes and spleen are essential for antigen presentation in vascularized cardiac allografts (Larsen et al. 1990; Lakkis et al. 2000). In addition, allorecognition may also occur in the allograft (Kreisel et al. 2002; Baddoura et al. 2005).

The transplanted heart is recognized as foreign through two distinctive pathways (Figure 3). The direct antigen recognition pathway involves recognition of intact allogeneic MHC expressed by donor APC – also called passenger leukocytes – that come along with the transplanted heart. The indirect allorecognition pathway involves the recognition of processed donor-derived peptides presented by recipient APC (Whitelegg and Barber 2004). The direct antigen presentation pathway elicits strong alloimmune activation early after transplantation (Benichou et al. 1999) and declines gradually possibly related to the gradual loss of donor-derived dendritic cells (Lechler and Batchelor 1982). In contrast, the indirect allorecognition pathway may be responsible for late rejection episodes and chronic rejection (Hornick et al. 2000; Whitelegg and Barber 2004). In addition to the professional APC, also allograft EC may directly activate CD8 cell-mediated rejection (Kreisel et al. 2002; 2004). MCH molecules
Table 2. Classification of rejection

<table>
<thead>
<tr>
<th>Rejection grade / AMR</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 0 R</td>
<td>No rejection</td>
</tr>
<tr>
<td>Grade 1 R, mild</td>
<td>Interstitial and/or perivascular infiltrate with up to 1 focus of myocyte damage</td>
</tr>
<tr>
<td>Grade 2 R, moderate</td>
<td>Two or more foci of infiltrate with associated myocyte damage</td>
</tr>
<tr>
<td>Grade 3 R, severe</td>
<td>Diffuse infiltrate with multifocal myocyte damage ± edema ± hemorrhage ± vasculitis</td>
</tr>
<tr>
<td>AMR 0</td>
<td>Negative for acute antibody-mediated rejection (AMR) No histologic or immunopathologic features of AMR</td>
</tr>
<tr>
<td>AMR 1</td>
<td>Positive for AMR Histologic features of AMR Positive immunofluorescence or immunoperoxidase staining for AMR (positive CD68, C4d)</td>
</tr>
</tbody>
</table>

The International Society for Heart and Lung Transplantation (ISHLT) standardized cardiac biopsy grading for acute cellular rejection and recommendations for acute antibody-mediated rejection. From ISHLT consensus report (Stewart et al. 2005). AMR, acute antibody-mediated rejection.

are the main transplant antigens that are recognized as foreign. They normally transfer endogenous and exogenous peptides from inside the cell to the cell surface for immune surveillance. MHC class I molecules are expressed by almost all nucleated cells and preferentially bind cytoplasmic and nuclear peptides (Daar et al. 1984a). In contrast, MHC class II molecules are mainly expressed by APC – but also by activated EC – and bind surface and extracellular proteins (Daar et al. 1984b). CD4 lymphocytes interact with MHC class II molecules (Doyle and Strominger 1987) whereas CD8 lymphocytes interact with MHC class I molecules (Norment et al. 1988). The MHC molecules are highly polymorphic and the MHC-disparity between the donor and recipient is the main determinant for allorecognition. The human leukocyte antigen (HLA) system encodes human MHC genes. HLA-A, HLA-B and HLA-C genes encode for class I molecules whereas HLA-DR, -DP and –DQ genes encode for class II molecules (Male et al. 2006).

The MHC molecules are not the only transplant antigens as also other polymorphic proteins – minor histocompatibility antigens – such as Y chromosome-encoded proteins in male to female transplantation can initiate allorecognition (Zelenika et al. 1998). Also, non-polymorphic proteins such as vimentin (Dunn et al. 1992; Jurcevic et al. 2001), cardiac myosin or collagen that are exposed during allograft damage may induce an autoimmune-like reaction against the transplanted organ and contribute to the rejection process (Rose 2005).

2.4. Lymphocyte activation

The change from a naïve T cell into an activated alloreactive cell involves three distinct signals (Figure 4) (Lindenteld et al. 2004a; Male et al. 2006). First, the alloantigen-MHC complex of the APC interacts with the complex of TCR, CD3, and CD4 or CD8 on the T lymphocyte. Second, co-stimulatory signals such as the interplay of CD28 on T cells and B7 (CD80 and CD86) on APC are needed (Turka et al. 1992). If the proper co-stimulatory signals are present, cytosolic calcineurin (Borel et al. 1976) activates the transcription factor nuclear factor of activated T cells (NF-AT), and also activated glucocorticoid receptors translocate into the nucleus. This initiates transcription of interleukin-2 (IL-2) and other pro-inflammatory cytokines. Third, autocrine and paracrine pro-inflammatory signals – most importantly IL-2 – are needed (Kirkman et al. 1985). Signalling from the cell surface receptor IL-2R, and subsequently from the cytoplasmic receptor mammalian target of rapamycin (mTOR) (Dumont et al. 1990) initiates the cell cycle...
that in lymphocytes involves *de novo* purine synthesis (Morris *et al.* 1990), and results in clonal proliferation of the alloreactive T cell (Lindenfeld *et al.* 2004a).

![Diagram of allorecognition](image)

**Figure 3.** Allorecognition occurs through direct (left) or indirect (right) pathways. In direct allorecognition, recipient T cells recognize intact allogeneic MHC expressed by donor APC. In indirect allorecognition, recipient T cells recognize processed transplant antigens presented by MHC of recipient APC. APC, antigen-presenting cell; MHC, major histocompatibility complex; TCR, T cell receptor. Modified from Whitelegg and Barber 2004.

### 2.5. Effector phase

Both innate and adaptive immunity participate in the injury and destruction of the transplanted organ. This effector phase involves cytotoxic T cells, delayed-type hypersensitivity and humoral responses (Figure 5) (Rocha *et al.* 2003). Alloreactive CD8 cytotoxic cells play a key role in mediating allograft injury. These cells may be primed through the direct allorecognition pathway by donor APC and also by donor EC (Kreisel *et al.* 2002). Alloreactive CD8 cells then identify the MHC class I on target cells, and elicit cytotoxic and apoptotic effects through the release of cytotoxic granules containing perforins and granzymes, and Fas ligand (Rocha *et al.* 2003). Although CD4 lymphocytes may also act as direct effector cells, they primarily function as helper cells for other immune cells. Three different classes of CD4 helper cells have been characterized and are associated with distinct cytokine expression profiles. CD4 Th1 helper cells primarily mediate the delayed-type hypersensitivity response by releasing pro-inflammatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). These cytokines further activate monocytes and macrophages by amplifying the production of pro-inflammatory cytokines. Soluble inflammatory mediators also increase vascular permeability that facilitates leukocyte extravasation to the inflamed area (Rocha *et al.* 2003).

Humoral response is initiated by CD4 Th2 helper cells that secrete IL-4, IL-5, IL-10 and IL-13. This results in the activation of alloreactive B cells that start to secrete antibodies directed to donor antigens. The main targets of the anti-donor antibodies are ABO and MHC antigens expressed by the allograft endothelium. The humoral response may result in antibody-
Lymphocyte activation

Figure 4. T cell activation involves three distinct signals. Allorecognition is initiated by the binding of alloantigen-MHC complex of the APC to the TCR of the T lymphocyte (1). If co-stimulatory signals (2) are present, intracellular signalling cascades lead to transcription of inflammatory cytokines. Autocrine and paracrine IL-2 signalling through IL-2R (3) activates mTOR and cell cycle, and results in T cell proliferation. Clinically-used immunosuppressive drugs and their targets are shown in red. APC, antigen-presenting cell; ATGAM, anti-thymocyte globulin; AZA, azathioprine; BAS, basiliximab; DAC, daclizumab; GR, glucocorticoid receptor; MHC, major histocompatibility complex; MMF, mycophenolate mofetil; NF-AT, nuclear factor of activated T cells; OKT, monoclonal muromonab CD3; TCR, T cell receptor; mTOR, mammalian target of rapamycin. Modified from Lindenfeld et al. 2004.

dependent cellular cytotoxicity mediated by macrophages and natural killer (NK) cells. These cells detect the anti-donor antibodies with their Fc-receptors and release perforins and inflammatory mediators. NK cells also function through “missing-self” signals that may result in immediate cytotoxic responses. In addition to the antibody-dependent cellular cytotoxicity, the binding of alloantibodies to the donor endothelium activates complement and coagulation cascades leading to widespread intravascular thrombosis, haemorrhage and tissue injury (Rocha et al. 2003; Colvin and Smith 2005). In addition to Th1 and Th2 helper cells, some CD4 cells adopt a phenotype that represses the alloimmune response. These regulatory CD4 cells (T_{reg}) express CD25 and the transcription factor Foxp3. The pathways resulting in the production of T_{reg} are not completely understood but have important implications for establishing tolerance to the transplanted allograft (Hara et al. 2001; Cobbold et al. 2004).

2.6. Endothelial cells and chemokines in leukocyte trafficking

Allorecognition, lymphocyte activation and the final effector phase all require that the inflammatory cells involved home into proper compartments. EC-leukocyte interactions and chemokines regulate the trafficking of cells in or out of the allograft, or the secondary lymphoid tissue (Figure 6A). Leukocyte extravasation to the transplanted heart occurs mainly in capillaries and post-capillary venules (Turunen et al. 1995). In lymph nodes, specialized post-capillary venules called high endothelial venules (HEV) are involved in leukocyte trafficking to secondary lymphoid tissue (Chin et al. 1991).

Leukocyte extravasation from the blood to the target tissue is a multi-step process (Figure 6B) (Sackstein 2005). First, leukocytes are tethered and start to roll on the endothelial layer mainly through the interaction of selectins and their ligands. The most important selectins are L-selectin on lymphocytes, and E-selectin and P-selectin on the surface of EC. In addition, the EC amine oxidase vascular adhesion molecule-1 (VAP-1) is important in leukocyte rolling (Salmi et al. 2001). Next, leukocytes are activated by chemokines released from the target
Clinical use of immunosuppressive drugs mainly target the different steps in the T cell activation pathway as illustrated in Figure 4. Polyclonal (ATGAM and Thymoglobulin) and monoclonal (OKT) anti-lymphocyte antibodies, and anti-IL-2R antibodies (basiliximab and daclizumab) are used for induction therapy that results in intense perioperative immunosuppression (Lindenfeld et al. 2004a). Maintenance triple-drug immunosuppression normally consists of a combination of calcineurin inhibitor (CsA or tacrolimus), anti-metabolite (azathioprine, AZA or mycophenolate mofetil, MMF) or proliferation signal inhibitor (sirolimus or everolimus), and corticosteroids. Anti-rejection therapy aims to reverse the rejection episode and may include the
increase or change of maintenance therapy, pulse corticosteroids, and anti-lymphocyte regimens (Lindenfeld et al. 2004a). Treatment options against humoral rejection are limited, and may include plasmapheresis, intravenous immunoglobulin or anti-CD20 antibody rituximab that results in transient B cell depletion (Colvin and Smith 2005).

The common side-effects of immunosuppressive drugs – malignancies and infections – are related to the inability of the host immune system to perform normal surveillance against foreign pathogens and malignant cells. In addition, anti-lymphocyte preparations may cause allergic reactions and cytokine release syndrome (Lindenfeld et al. 2004a). Corticosteroids are associated with multiple long-term metabolic and cosmetic adverse effects (Lindenfeld et al. 2004b). Both calcineurin inhibitors are nephrotoxic. CsA often results in hypertension, hyperlipidemia, hirsutism, gingival hyperplasia and neurological symptoms whereas tacrolimus more commonly induces diabetes and neurological symptoms (Lindenfeld et al. 2004b). Azathioprine and mycophenolate mofetil both target the de novo purine synthesis pathway that is essential for cell cycle and lymphocyte proliferation. Azathioprine may induce myelosuppression resulting in anemia, leukopenia and thrombocytopenia. Mycophenolate mofetil induces mainly gastrointestinal side-effects but may also result in myelosuppression. Sirolimus and everolimus both inhibit the intracytosolic enzyme mTOR that is involved in the cell cycle regulation. In addition to lymphocytes, mTOR inhibitors also regulate the proliferation of arterial EC and SMC. The major side-effects for sirolimus are hyperlipidemia, thrombocytopenia and neutropenia, and everolimus has similar adverse effects (Lindenfeld et al. 2004b).

3. Cardiac allograft arteriosclerosis

Cardiac allograft arteriosclerosis i.e. transplant coronary artery disease (TxCAD) – an accelerated form of arteriosclerosis – is a major limitation for long-term survival of heart transplant patients. “Cardiac allograft vasculopathy” and “transplant vasculopathy” have also been used to describe the disease. TxCAD probably plays a causal role in the development of other manifestations of chronic rejection as parenchymal fibrosis. Also, intimal thickening in allograft arteries is a common histomorphological finding in chronic rejection of other solid organ transplants such as kidney, liver and lung (Hayry et al. 1993; Libby and Pober 2001).
3.1. Diagnosis, incidence and prognosis

Due to denervation of the transplanted heart, patients with TxCAD do not usually experience chest pain. Instead, the clinical symptoms of TxCAD are mostly related to heart failure and may include fatigue, impaired exercise tolerance (Schwaiblmair et al. 1999) and ventricular arrhythmias. TxCAD is usually diagnosed invasively but also non-invasive methods have been developed. The most common diagnostic tool is angiography but it underestimates the disease when compared to histological sections (Johnson et al. 1991). Intravascular ultrasound (IVUS) is more sensitive than angiography (St Goar et al. 1992). An example of angiographically silent TxCAD that is detected with multi-vessel IVUS is presented in Figure 7. TxCAD may also be diagnosed by histology of explanted transplants, dobutamine stress echocardiography (Spes et al. 1999), multi-slice computer tomography (Romeo et al. 2005) or magnetic resonance imaging (Caus et al. 2006).

![Figure 7. Detection of TxCAD with angiography and intravascular ultrasound (IVUS, left) and a histological presentation (right). The heart transplant patient had a normal angiography as shown in the background. Multivessel IVUS showed an eccentric intimal thickening in the right coronary artery (RCA), whereas left anterior descending (LAD) and left circumflex (LCx) coronary arteries were intact. White arrows indicate the IVUS imaging sites. Reprinted from Kapadia et al. 2000 with permission from the International Society for Heart and Lung Transplantation. Histological presentation of TxCAD of another heart transplant patient on the right (kindly provided by Dr Kaisa Salmenkivi, University of Helsinki).](image)

The incidence of angiographically-diagnosed TxCAD is 42% at five years (Costanzo et al. 1998). Using multi-vessel IVUS, 58% of heart transplant patients have TxCAD at 1 year, 71% at 2 years, and 74% at 3 years (Kapadia et al. 2000). Intimal thickening diagnosed by IVUS predicts later angiographic TxCAD, and is associated with impaired survival even in patients with normal angiography (Mehra et al. 1995b; Rickenbacher et al. 1995). Progression of intimal thickening during the first year after heart transplantation detected with IVUS is a reliable surrogate marker for subsequent adverse events (Kobashigawa et al. 2005; Tuzcu et al. 2005) as shown in Figure 8.

3.2. Immunological and non-immunological risk factors

Both immunological and non-immunological risk factors for TxCAD have been characterized. Moderate and severe acute rejections have a cumulative impact on the onset of TxCAD (Stoica et al. 2006) and the presence of HLA-directed antibodies after heart transplantation is associated with impaired graft outcome (Tambur et al. 2005). In addition, the panel-reactive antibody test reactivity >20% (Taylor et al. 2003) and anti-EC antibodies (Dunn et al. 1992; Jurcevic et
al. 2001) are risk factors for TxCAD. Non-immunological TxCAD risk factors include both transplantation-related and traditional cardiovascular risk factors. A heart received from a living donor due to a domino heart transplantation results in less TxCAD than from donors that have suffered brain death (Anyanwu et al. 2003). Also, violent donor brain death mechanism results in accelerated TxCAD and reduced graft survival (Mehra et al. 2004). Cytomegalovirus (CMV) infection of transplant patients is a risk factor for TxCAD (Grattan et al. 1989). Also, traditional cardiovascular risk factors such as hyperlipidemia (Escobar et al. 1994), abnormal glucose metabolism (Valantine et al. 2001a), hypertension and smoking (Radovancevic et al. 1990) have an impact on the development of TxCAD.

3.3. Pathological findings

Histological analysis of transplanted hearts has revealed that TxCAD differs from traditional coronary artery disease (CAD) as schematically illustrated in Figure 9. CAD usually affects proximal coronary arteries, the plaques are mostly focal and eccentric, and consist of lipids and calcium deposits. In addition, there is usually no adventitial inflammation or fibrosis, and outward remodelling may be present (Arbustini and Roberts 1996). In contrast, TxCAD affects both large and small coronary arteries (Lin et al. 1994), the neointimal plaques are diffuse and concentric, they consist of both cellular and fibrous tissue, and rarely of calcium deposits or cholesterol. Also, adventitial inflammation and fibrosis are usually present and may constrict the affected arteries (Arbustini and Roberts 1996). In arteries with cellular lesions, the lesions contain T lymphocytes, monocytes and SMC (Liu and Butany 1992). IVUS studies have indicated that pre-existing coronary artery disease of the donor does not accelerate the subsequently-developing TxCAD suggesting that the underlying pathophysiology of the two diseases is different (Botas et al. 1995). Also, serial IVUS investigations show that the intimal thickening in TxCAD occurs early after heart transplantation whereas arterial constriction is the predominant factor in the luminal loss late after transplantation (Tsutsui et al. 2001).

3.4. Response-to-injury hypothesis

TxCAD can be viewed as a response-to-injury resulting in activation and dysfunction of EC and SMC (Hayry et al. 1993). This in turn leads to vascular remodelling and intimal development.
Figure 9. Difference between coronary artery disease, CAD (A) and TxCAD (B). CAD is characterized by focal and eccentric localization to proximal epicardial coronary arteries. Some inflammatory cells, foam cells and SMC, and calcium deposits are present in the fibrous and cholesterol plaque. Minimal adventitial inflammation or fibrosis is detected and some outward remodelling may occur (A). In contrast, TxCAD is characterized by diffuse and concentric localization to both large epicardial and small intramyocardial coronary arteries. The intima consists of SMC, inflammatory cells and fibrosis. There is prominent adventitial inflammation and fibrosis that may constrict the affected artery (B). EC, endothelial cell; SMC, smooth muscle cell.

Figure 10. A schematic time line of the development of TxCAD. Both non-immunological and immunological damage to the transplanted heart (blue) result in a poorly regulated fibroproliferative response (red) that results in TxCAD and involves dysfunction of allograft EC and SMC. Continuous injury, or intermittent injury and response-to-injury cycles may be involved. Ab, antibody; CMV, cytomegalovirus; CMC, cardiomyocyte; EC, endothelial cell; I/R, ischaemia-reperfusion; SMC, smooth muscle cell.
similarly as proposed for atherosclerosis by Russell Ross (Ross 1993). A schematic timeline for the development of the disease, and factors involved in allograft injury and fibroproliferative response are shown in Figure 10. In heart transplantation, the peri- and post-operative immunological and non-immunological factors described above may damage the allograft. Although the immunological mechanisms generally predominate, also non-immunological factors may modify the alloimmune response and directly damage the allograft. The continuous low grade damage to cardiac allograft arteries and EC in turn triggers the fibroproliferative phase of TxCAD that leads to the accumulation of SMC in the intima of affected arteries (Libby and Pober 2001). Numerous regulatory molecules have been implicated in TxCAD and some of these factors are listed in Table 3. The origin of intimal SMC is probably heterogenous and reflects the intensity of injury as discussed below and shown in Figure 11.

3.5. Endothelial cells in cardiac allograft arteriosclerosis

Functional endothelium inhibits thrombus formation, leukocyte adhesion and SMC proliferation. In transplanted hearts, endothelial dysfunction plays a central role in the development of TxCAD (Valantine 2003). Endothelial dysfunction is related to the gradual loss of endothelial nitric oxide synthase (eNOS) (Vejlstrup et al. 2002) and predicts the later development of TxCAD (Hollenberg et al. 2001; Marti et al. 2001). Activated and dysfunctional endothelium may enhance cardiac allograft inflammation by inducing antigen presentation (Kreisel et al. 2002), adhesion protein expression (Labarrere et al. 1997) and chemokine expression (Kobayashi et al. 2003). In addition, EC activation – together with adherent platelets – may induce migration and proliferation of SMC and their progenitors by secreting potent SMC cytokines (Hosenpud et al. 1996; Fateh-Moghadam et al. 2000). Antibodies directed against allograft EC are important in the development of TxCAD (Dunn et al. 1992; Jurcevic et al. 2001).

The origin of cardiac allograft vascular cells has mechanistic significance and also implications for therapeutic approaches. Although endothelial chimerism was proposed early and was considered beneficial for the acceptance of the allograft (Medawar 1965), current data indicate that the replacement of allograft EC with recipient cells is a rare phenomenon (Koopmans et al. 2006). Experimental studies show that the endothelium of aortic allografts – that undergo extensive injury – is largely replaced with recipient-derived EC (Hillebrands et al. 2001; 2002). In contrast, endothelial replacement in experimental cardiac allografts is uncommon probably reflecting the lower intensity of injury compared to aortic allografts (Hillebrands et al. 2001). In clinical situation, female-to-male heart transplantations allow the detection of recipient cells in the allograft although there are methodological problems (Koopmans et al. 2005). Using the female-to-male heart transplantation samples, cardiac allograft EC were shown to originate from the donor and not from the recipient (Hruban et al. 1993). In contrast, another study estimated that 7% of allograft capillaries contained the male chromosome (Quaini et al. 2002). In this study the recipient atrium – that is preserved in the operation – was suggested as a potential source for stem cells that could participate in cardiac chimerism. The presence of circulating progenitor cells was not investigated. Collectively these reports indicate that replacement of EC with recipient-derived cells is rare in transplanted hearts. Also, human kidney transplantation studies indicate that allograft chimerism does not account for the long-term acceptance of transplanted organs (Mengel et al. 2004; van Poelgeest et al. 2005).
3.6. Smooth muscle cells in cardiac allograft arteriosclerosis

Accumulation of SMC into the intima of cardiac allograft arteries is a predominant factor in the fibroproliferative response to allograft damage (Arbustini and Roberts 1996). In chronic rejection, the phenotype of allograft SMC changes from the normal contractile to a more primitive phenotype with proliferative capacity (Suzuki et al. 1996; Autieri 2003). Several transcription factors, cell cycle molecules and growth factors regulate the proliferative SMC response in TxCAD as listed in Table 3.

### Table 3. Factors with a potential role in TxCAD

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<td>Chemokines</td>
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AIF-1, allograft inflammatory factor-1; ET-1, endothelin-1; MIG, monokine induced by interferon; I-TAC interferon-inducible T cell alpha chemoattractant; TSP-1, thrombospondin-1; other abbreviations are listed on page 8.
Traditionally, intimal SMC were considered to migrate from the media of the affected artery (Hayry et al. 1993) but this view has been recently challenged. The origin of intimal cells in cardiac allografts is probably heterogenous as shown in Figure 11. Experimental studies show that the intima of aortic allografts mainly consists of recipient-derived (Hillebrands et al. 2001) and recipient bone marrow (BM)-derived cells (Shimizu et al. 2001). In heterotopic cardiac allografts, most of the intimal SMC have been shown to originate from the recipient (Hillebrands et al. 2001; Saiura et al. 2001) and some from the recipient BM (Sata et al. 2002; Saiura et al. 2003).

![Figure 11](image)

**Figure 11.** Possible origin of intimal cells in cardiac allografts. The origin of intimal SMC in cardiac allografts is probably heterogenous reflecting the injury to the transplanted organ. Mature donor SMC may migrate from the media to the intima, or mature EC may transdifferentiate to SMC. In addition, progenitor cells from the donor and the recipient may be recruited to the intima and differentiate to SMC. BM, bone marrow; EC, endothelial cell; SMC, smooth muscle cell.

In a clinical study, recipient-derived SMC were not found in transplanted hearts (Hruban et al. 1993). Another study reported that on the average 2.6% (approximately 7.5% if the sensitivity of the assay is taken into account) of intimal SMC in small to medium size coronary arteries originated from the recipient (Glaser et al. 2002). In a study that excluded the neointimal areas, 10% of SMC+ arterioles had the male Y-chromosome indicating recipient origin (Quaini et al. 2002). In contrast, when the origin of SMC in the intima of large epicardial arteries was investigated, all intimal SMC were found to originate from the donor and not from the recipient (Atkinson et al. 2004). In conclusion, the origin of cardiac allograft neointimal cells may be heterogenous and reflect the ability of donor-derived SMC or their progenitors, and recipient SMC or their progenitors, to participate in reparative processes in different models and different intensity of injury. Therefore, although recipient-derived SMC may be a predominant source of intimal cells in experimental models with extensive injury, current clinical evidence suggests minimal contribution of recipient-derived SMC in TxCAD (Glaser et al. 2002; Hillebrands et al. 2003; Atkinson et al. 2004). Similarly, in mechanical injury models, recipient BM-derived cells substantially contribute to the accumulation of intimal SMC after wire injury, but the intimal SMC originate from non-BM sources after cuff-mediated periadventitial injury or carotid artery ligation (Tanaka et al. 2003).
3.7. Current clinical therapeutic options

The current clinical tools for preventing the development of TxCAD and in particular for treatment of the established disease are limited. The progression of TxCAD is slowed in patients randomized to receive MMF instead of AZA (Kobashigawa et al. 2006). mTOR inhibitors everolimus (Eisen et al. 2003) and rapamycin (Mancini et al. 2003) also slow the progression of TxCAD. The effects of MMF and particularly mTOR inhibitors may be in part due to their direct anti-proliferative and anti-migratory effects on SMC (Marx et al. 1995; Raisanen-Sokolowski et al. 1995; Poon et al. 1996). Experimental results also suggest that treatment with rapamycin may even reverse the established vascular disease in cardiac allografts (Poston et al. 1999). In addition to the clinically-used immunosuppressive drugs, statins, calcium channel blockers and anti-CMV-regimen regulate the progression of TxCAD. The use of calcium channel blockers or angiotensin-converting enzyme inhibitors decrease TxCAD detected with IVUS one year after the heart transplantation (Mehra et al. 1995a). Also, the use of calcium channel blockers decreases angiographically detected TxCAD two years after transplantation (Schroeder et al. 1993). Simvastatin (Wenke et al. 1997; Wenke et al. 2003) and pravastatin (Karamlou et al. 2005) have beneficial effects on the development of TxCAD. In addition to their cholesterol lowering effects, statins also mediate cholesterol-independent beneficial endothelial effects and direct anti-inflammatory effects (Weis et al. 2001; Shimizu et al. 2003). In CMV seropositive patients, profylaxis with gangciclovir reduces TxCAD (Valantine et al. 1999), and additional treatment with CMV hyperimmune globulin may be helpful (Valantine et al. 2001b).

Currently, no medical treatment to reverse established TxCAD is available. The use of angioplasty is limited due to the diffuse nature of the disease and the high restenosis rate (Musci et al. 1998). Retransplantation remains the only effective treatment option for established TxCAD but the long-term survival is lower after retransplantation than after primary transplantation (Topkara et al. 2005).

4. Angiogenesis

4.1. Function and structure of the vascular system

The vascular system is divided into arteries, capillaries, veins and lymphatics by functional and structural differences. Arteries form a large blood-conducting network from heart to peripheral tissues. Large arteries are elastic and balance the high pulse pressure, medium size arteries have bigger muscular coating, and small arteries and arterioles regulate blood flow at tissue level. Capillaries are thin vessels that are important in the transfer of oxygen, nutrients and waste products between blood and tissue. Most of leukocyte traffic from blood to tissue occurs after capillaries in post-capillary venules. Small veins gradually collate into bigger veins and flow back to the heart (Ross 2005). In contrast to blood circulation, the lymphatic system forms a one-way system that transports extravasated protein-rich fluids and leukocytes from the tissue to lymphatic vessels, and finally to the venous circulation through the thoracic duct. Lymphatic vessels pass through lymph nodes that are important for immunosurveillance (Alitalo et al. 2005; Ross 2005).

The general structure of blood vessels consists of three different layers – tunica intima, tunica media and tunica adventitia. The inner layer – tunica intima – is formed of a single cell layer of EC, basal lamina of the endothelium, and a subendothelial layer that contains loose connective
tissue and SMC. The middle layer – *tunica media* – forms the muscular layer of vessels and mainly contains SMC. *Tunica media* is separated from *tunica intima* by *lamina elastica interna*, and from *tunica adventitia* by *lamina elastica externa*. The outer layer – *tunica adventitia* – consists mainly of loose fibrous tissue that connects the vessel to the surrounding tissue. Small blood vessels – *vasa vasorum* – that provide nutrients to the vessel wall are also present in the adventitia (Ross 2005).

The heart receives its blood supply through coronary arteries. The left and right coronary artery branch off the aorta above the aortic valve, form large epicardial arteries, feed the myocardium through smaller intramyocardial branches and finally through myocardial capillaries. In normal human heart, the *tunica intima* is thick in large epicardial coronary arteries and progressively thinner in smaller intramyocardial arteries (Ross 2005). In contrast to human arteries, the normal intimal layer of both large and small arteries of rodents is thin and devoid of subendothelial tissue (Du Toit *et al*. 2001; Khan *et al*. 2006). In human atherosclerosis, the intima of large epicardial coronaries – especially in bifurcations – is susceptible for eccentric thickening, lipid accumulation and progression of the disease. In contrast, neointimal formation in TxCAD is concentric and affects the whole arterial network from large epicardial to small intramyocardial vessels (Arbustini and Roberts 1996).

The capillaries and post-capillary venules only contain the endothelium, basal lamina of the endothelium, and some supporting pericytes. In contrast to SMC, pericytes share the same basement membrane with EC, and they may have direct contacts with the endothelium through holes in the basement membrane (Armulik *et al*. 2005; Ross 2005). Transfer of inflammatory cells from blood vessels to the transplanted heart occurs mainly in capillaries and post-capillary venules (Turunen *et al*. 1995). Also, as intimal and adventitial angiogenesis is seen in TxCAD (Atkinson *et al*. 2005; Seipelt *et al*. 2005), the function of both microvessels and arteries may be important in cardiac allograft inflammation and arteriosclerosis.

### 4.2. Vessel formation and maturation

Mice that lack only one allele of vascular endothelial growth factor (VEGF) die *in utero* demonstrating the complex genetic code involved in the development of normal vasculature (Carmeliet *et al*. 1996; Ferrara *et al*. 1996). In addition to VEGF, many other growth factors and a delicate communication between multiple cell types like EC and the surrounding supporting cells and matrix, are needed for normal vascular formation and maintenance (Hellstrom *et al*. 2001). In contrast to embryonic and growing vessels, adult vasculature is usually in a quiescent state perturbed only by wound healing, adaptive responses to tissue hypoxia, reproductive functions and inflammation. Improper regulation of angiogenesis is also involved in a variety of pathological conditions. Inadequate angiogenesis is harmful in situations with tissue ischemia, whereas the opposite is true for tumour growth and many inflammatory conditions (Yancopoulos *et al*. 2000; Ferrara *et al*. 2003).

Vascular development is believed to occur through different mechanisms. Vasculogenesis refers to a process where vascular stem and progenitor cells such as angioblasts differentiate to EC and form primitive vascular structures as shown in Figure 12. In contrast, angiogenesis refers to a process where existing vascular network grows by sprouting, bridging or intussusceptive growth. Generally, vasculogenesis occurs before angiogenesis but the two phenomenons overlap (Yancopoulos *et al*. 2000; Ferrara *et al*. 2003). For a long time, vasculogenesis was
thought to occur only during embryonic development but it may also occur in adults (Asahara et al. 1999).

Vessel maturation is a process where the primitive endothelial tubes are stabilized by recruitment of supporting mural cells and generation of ECM. Vessel maturation is regulated by physical forces and growth factors, and it involves delicate interactions between EC and the surrounding cells. A schematic presentation of vasculogenesis and blood vessel maturation is given in Figure 12.

![Figure 12: Vasculogenesis and blood vessel maturation.](image)

**Figure 12.** Vasculogenesis and blood vessel maturation. In vasculogenesis, vascular progenitor cells angioblasts migrate and proliferate, and form primitive endothelial tubes involving VEGF and its receptors VEGFR-1 and -2. The endothelial cells recruit pericytes and SMC and their progenitors involving PDGF-B/PDGFR-β and Ang1/Tie2 signalling. TGF-β is important in the further maturation of the blood vessel. Ang, angiopoietin; EC, endothelial cell; PDGF, platelet-derived growth factor; SMC, smooth muscle cell; TGF, transforming growth factor; VEGF, vascular endothelial growth factor. Modified from Cleaver and Melton 2003.

4.3. Vascular growth factors

The existence of angiogenic factors was first postulated when enhanced vascularization was seen in tumours. Later, a soluble factor that induced capillary formation was found from tumours (Folkman et al. 1971). Since then, many molecules have been implicated as positive regulators of angiogenesis such as fibroblast growth factors (FGF), transforming growth factors (TGF), hepatocyte growth factor (HGF), tumor necrosis factor-α (TNF-α), IL-8 and angiogenin (Ferrara et al. 2003).

This study focused on VEGF, angiopoietin and PDGF growth factor families because of their importance in angiogenesis, inflammation and fibroproliferative diseases, and thus their possible regulatory roles in the development TxCAD. Schematic presentation of their function, structure, ligands and receptors is given in Figures 13-15. All ligands of these three growth factor families form dimers. The corresponding receptors dimerize upon ligand binding, phosphorylate tyrosine residues in the cytosol, and activate intracellular signalling pathways (Jones et al. 2001a; Ferrara et al. 2003; Fredriksson et al. 2004; Alitalo et al. 2005).

4.3.1. The VEGF family

VEGF was first discovered as a tumour-secreted factor that induced vascular permeability and was first named accordingly as vascular permeability factor (VPF) (Senger et al. 1983; Ferrara
The VEGF family currently consists of six known ligands: PlGF, VEGF (VEGF-A), VEGF-B, VEGF-C, VEGF-D and VEGF-E. The receptors for the VEGF family ligands include VEGFR-1, VEGFR-2, VEGFR-3 and neuropilins (NRP-1 and NRP-2) (Ferrara et al. 2003; Tammela et al. 2005). The binding properties of VEGF ligands and the principal effects of VEGF receptors are summarized in Figure 3.

4.3.1.1. VEGF

VEGF (VEGF-A) binds to VEGFR-1, VEGFR-2, NRP-1 and NRP-2 and is considered to be the major regulator of the angiogenic switch. Alternative exon splicing of the VEGF gene results in different splicing variants that differ in their heparin binding affinities. Splicing variant VEGF<sub>121</sub> does not bind to heparin, whereas VEGF<sub>165</sub> and VEGF<sub>206</sub> are almost completely sequestered in the ECM, and the predominant isoform VEGF<sub>165</sub> has intermediate properties (Houck et al. 1991; Tischer et al. 1991; Ferrara et al. 2003).

VEGF is produced in a variety of adult tissues, vascular cells like EC and SMC, and inflammatory cells like monocyte/macrophages, neutrophils and lymphocytes (Tomizawa et al. 1985; Ferrara et al. 1991; Berse et al. 1992; Freeman et al. 1995; McCourt et al. 1999; Melter et al. 2000). Oxygen tension is a major regulator of VEGF expression and involves the activation of hypoxia-inducible factor-1 (HIF-1) transcription factor system. Hypoxia stabilizes HIF-1, which activates transcription of hypoxia response element (HRE)-possessing genes such as VEGF (Shweiki et al. 1992; Liu et al. 1995). In addition to oxygen tension, several growth factors as epidermal growth factor (EGF), TGF-α, TGF-β, keratinocyte growth factor, insulin-like growth factor-1, FGF and PDGF may induce VEGF expression (Ferrara et al. 2003). VEGF induction is also linked to inflammation through several pro-inflammatory cytokines such as MCP-1 (Parenti et al. 2004; Hong et al. 2005), TNF-α (McCourt et al. 1999), and binding of CD40L of activated T-cells (Melter et al. 2000) upregulate VEGF expression.

VEGF elicits its functions primarily on EC, but has also direct receptor-mediated effects on inflammatory, hematopoietic, stem and progenitor cells, and SMC (Clauss et al. 1990; Barleon et al. 1996; Clauss et al. 1996; Hattori et al. 2001; Ishida et al. 2001). VEGF promotes migration, proliferation and survival of EC, and EC production of nitric oxide (NO) and proteinases involved in matrix degradation (Pepper et al. 1991; Unemori et al. 1992; Ferrara et al. 2003). Mice with only one deficient VEGF allele die in utero demonstrating the importance of VEGF in EC functions and vascular development (Carmeliet et al. 1996; Ferrara et al. 1996). In contrast, mature and quiescent adult vasculature is largely resistant to VEGF inhibition (Gerber et al. 1999; Kamba et al. 2005). Systemic administration of VEGF results in NO dependent arterial vasodilatation and hypotension (Ku et al. 1993; Yang et al. 1996).

VEGF has many pro-inflammatory properties because it induces expression of adhesion proteins, tissue factor and pro-inflammatory chemokines such as MCP-1 and IP-10 in EC (Kim et al. 2001b; 2002; Reinders et al. 2003b; Boulday et al. 2006). It also directly increases monocyte migration, and promotes vascular permeability (Barleon et al. 1996; Weis et al. 2004) and has a regulatory role in sepsis (Yano et al. 2006). VEGF-producing dendritic cells induce angiogenesis in lymph nodes (Webster et al. 2006) but VEGF does not have direct effects on T cell proliferation (Reinders et al. 2003b). VEGF has also effects on BM-derived cells as it is important in haematopoiesis, mobilization and endothelial differentiation of vascular progenitor cells, and mobilization of myeloid progenitor cells (Ferrara et al. 1996; Yamashita et al. 2000; Hattori et al. 2001; Luttun et al. 2002). Mobilization of endothelial
progenitor cells after acute myocardial infarction involves VEGF (Shintani et al. 2001), and VEGF-mediated endothelial repair halts the progression of restenosis (Asahara et al. 1995). In addition to these positive effects on endothelial repair and angiogenesis, VEGF has also harmful effects in vascular disease. In some experimental models VEGF enhances atherosclerosis by increasing macrophage migration and plaque EC content (Celletti et al. 2001a; 2001b) but this is not the case in all experimental models (Leppanen et al. 2005). Although VEGF primarily activates vascular EC, effects on lymphatic EC have also been reported (Nagy et al. 2002). Taken together, VEGF effects are not restricted only to EC and angiogenesis, but intervene with inflammation and vascular repair both in physiological and pathological situations.

**Figure 13.** VEGF ligands and receptors. VEGF ligands have different binding properties to VEGF receptors. VEGFR-1 modulates VEGF responses on EC and has chemotactic effects in monocytes and SMC. VEGFR-1 has also a functional role in haematopoiesis, and the soluble sVEGFR-1 inhibits VEGF effects. VEGFR-2 elicits the main mitogenic and pro-inflammatory effects on vascular EC but has a functional role also in haematopoietic and vascular progenitor cells. VEGFR-3 signalling principally regulates the development and functionality of lymphatic EC, and migration of antigen-presenting cells. EC, endothelial cell; HSC, haematopoietic stem cell; NO, nitric oxide; PI GF, placental growth factor; SMC, smooth muscle cell; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; sVEGFR, soluble VEGFR.
Placental growth factor (PIGF) belongs to the VEGF family and was first detected in human placenta (Maglione et al. 1991). PIGF binds to VEGFR-1 and NRP-1, and is not essential for normal vascular development (Carmeliet et al. 2001; Gigante et al. 2004). In contrast, PIGF may enhance the effects of VEGF particularly in pathological conditions (Park et al. 1994; Carmeliet et al. 2001).

VEGF has an autoregulatory role in PIGF induction on EC, showing a close relation to the regulation of the two growth factors (Zhao et al. 2004; Yao et al. 2005). Also, PIGF may directly phosphorylate VEGFR-1 which in turn activates VEGFR-2. PIGF/VEGF heterodimers induce VEGFR-1/VEGFR-2 heterodimer formation enhancing the angiogenic response compared to the effect by VEGF only (Autiero et al. 2003).

PIGF has a clear inflammatory role and it activates both hematopoietic stem cells (HSC) (Rafii et al. 2003) and myelomonocytic inflammatory cells through VEGFR-1 (Barleon et al. 1996; Clauss et al. 1996; Adini et al. 2002). PIGF expression in skin induces an angiogenic response with increased inflammation and oedema (Odorisio et al. 2002; Oura et al. 2003) and the arteriogenic properties of PIGF involve monocyte/macrophage activation (Pipp et al. 2003). Inhibition of PIGF receptor VEGFR-1 has been helpful in inflammatory conditions and atherosclerosis and probably relates to PIGF/VEGFR-1 effects on myelomonocyte cell recruitment (Luttun et al. 2002).

VEGF-B binds to VEGFR-1 and NRP-1 and is expressed in high levels in tissues with elevated metabolism, such as skeletal muscle and heart. Two splicing variants of VEGF-B with different affinities to heparin exist (Olofsson et al. 1996a; 1996b). Lack of VEGF-B does not impair vascular development, but it may yield to conducting defects and size reduction in the heart, and impaired recovery after myocardial ischemia (Bellomo et al. 2000; Aase et al. 2001). VEGF-B is a weak mitogen for EC (Olofsson et al. 1996a) but it may have some angiogenic potential in vivo (Silvestre et al. 2003; Mould et al. 2005). VEGF-B may also have a role in inflammatory angiogenesis as loss of VEGF-B protects against the development of arthritis (Mould et al. 2003).

VEGF-C binds to VEGFR-2 and VEGFR-3 and is a major regulator in the development of lymphatic vasculature (Jeltsch et al. 1997; Oh et al. 1997). Loss of one VEGF-C allele causes prominent lymphatic hypoplasia, whereas loss of both VEGF-C alleles results in embryonic lethality (Karkkainen et al. 2004). VEGF-C is expressed at sites of lymphangiogenesis during embryonic development and is expressed in heart, lung and kidney of adult mice (Kukk et al. 1996). In addition to regulating the growth of lymphatic vessels, VEGF-C is linked to inflammation. Its expression is upregulated by pro-inflammatory cytokines TNF-α, IL-1α and IL-1β (Ristimaki et al. 1998). Inflammatory cells such as macrophages and dendritic cells are a rich source of VEGF-C (Cursiefen et al. 2004; Baluk et al. 2005). VEGF-C has migratory effects on macrophages and dendritic cells (Skobe et al. 2001; Chen et al. 2004b). VEGF may recruit VEGF-C-expressing macrophages that contribute to inflammatory lymphangiogenesis.
(Cursiefen et al. 2004).

4.3.1.5. VEGF-D

Human VEGF-D binds to VEGFR-2 and VEGFR-3 whereas mouse VEGF-D may bind only to VEGFR-3 (Baldwin et al. 2001). Like VEGF-C, also VEGF-D undergoes proteolytical modification, is involved in lymphangiogenesis, and has angiogenic properties. Adenovirally delivered VEGF-D induces a potent angiogenic and lymphangiogenic response in skeletal muscle and is associated with elevated vascular permeability (Rissanen et al. 2003) but the relative effects on angiogenesis and lymphangiogenesis may depend on the tissue used (Byzova et al. 2002). In contrast to VEGF-C, VEGF-D is not essential for the development of lymphatic vessels (Baldwin et al. 2005).

4.3.1.6. VEGF-E

VEGF-E is a VEGF homologue that was discovered in the genome of parapoxvirus Orf. Dermal vascularization in sheep and humans infected with the Orf virus suggests the presence of virally-expressed VEGF-E (Lyttle et al. 1994). VEGF-E binds exclusively to VEGFR-2 and it has been used to investigate the relative roles of VEGF receptors (Shibuya 2003). The VEGFR-2-specific VEGF-E produces more angiogenesis in mouse skin than VEGFR-1-specific PI GF suggesting a more pronounced role for VEGFR-2 in mediating VEGF-induced angiogenesis (Kiba et al. 2003).

4.3.1.7. VEGFR-1

VEGFR-1 (also known as fms-like tyrosine kinase, Flt-1) is a receptor for VEGF, VEGF-B and PlGF. Like all VEGF receptors, VEGFR-1 has seven extracellular immunoglobulin-like domains, a transmembrane region, and an intracellular tyrosine kinase domain. After binding of the dimerized ligand, the receptor itself undergoes dimerization and the intracellular tyrosine kinase domain is phosphorylated (Ferrara et al. 2003). A soluble form of VEGFR-1 (sVEGFR-1) also exists, that has only the extracellular part of the receptor and inhibits VEGF action (Kendall and Thomas 1993; Ambati et al. 2006).

VEGFR-1 expression is found in EC, myelomonocytes, HSC, SMC, and vascular progenitor cells (Ferrara et al. 2003). VEGFR-1 expression is upregulated by hypoxia via HIF-1-mediated mechanisms (Gerber et al. 1997). The effect of VEGFR-1 on EC and angiogenesis is controversial and may be different depending on the stage of development and cell type. Loss of functional VEGFR-1 results in embryonal lethality (Fong et al. 1995) with excessive endothelial progenitor cell (EPC) proliferation and vascular disorganization (Fong et al. 1999). In addition, VEGFR-1 activity on EC does not require the tyrosine kinase domain of the receptor (Hiratsuka et al. 1998) suggesting that VEGFR-1 may act as a decoy receptor and a negative regulator of VEGF-induced vasculogenesis in embryos. VEGF induces only a weak VEGFR-1 autophosphorylation in EC (de Vries et al. 1992; Waltenberger et al. 1994), but VEGFR-1 ligands may enhance the angiogenic effects of VEGF by inducing VEGFR-1/VEGFR-2 interactions (Autiero et al. 2003).

VEGFR-1 has a functional role in many non-EC as it mediates the migration and survival of BM progenitor cells (Hattori et al. 2002; Rafii et al. 2003). VEGFR-1 provides also a migratory
stimulus for myelomonocytic cells indicating that VEGFR-1 plays a role in inflammatory conditions (Clauss et al. 1990; 1996; Barleon et al. 1996; Luttun et al. 2002). The arteriogenic properties of VEGFR-1 may rely on the recruitment of monocytes, too (Luttun et al. 2002; Pipp et al. 2003). VEGFR-1 is involved in cardioprotection after ischemic preconditioning (Addya et al. 1997; Luttun et al. 2002), consistent with impaired recovery of VEGF-B deficient mice after myocardial ischemia (Bellomo et al. 2000). It is normally expressed at low levels in vascular SMC but hypoxia and vascular injury upregulate its expression. This in turn may render SMC responsive for VEGFR-1-mediated migratory and proliferative signals, indicating a possible role for pathological SMC functions in vascular remodelling (Couper et al. 1997; Wang and Keiser 1998; Grosskreutz et al. 1999; Shibata et al. 2001; Parenti et al. 2002; 2004).

4.3.1.8. VEGFR-2

VEGFR-2 (also known as kinase-insert domain receptor, KDR/fetal liver kinase, Flk-1) binds VEGF, VEGF-C, VEGF-D (in humans) and VEGF-E, and is considered to elicit the majority of VEGF-induced mitogenic, angiogenic and permeability effects (Ferrara et al. 2003).

VEGFR-2 is mainly expressed in EC but also in HSC and endothelial progenitor cells (Eichmann et al. 1997). Unlike VEGFR-1, VEGFR-2 expression is not regulated by hypoxia (Gerber et al. 1997) but is autoregulated by VEGF, VEGF-C and VEGF-D (Tammela et al. 2005). VEGFR-2 expression is prominent during embryonal development and essential for haematopoiesis and vasculogenesis (Kaipainen et al. 1993; Shalaby et al. 1995). In adults, VEGFR-2 expression is usually downregulated and present only at sites of active angiogenesis such as wound healing, tumours, and after myocardial infarction (Shibuya 1995; Li et al. 1996). Accordingly, adult vasculature is largely VEGF-independent, although certain EC that have high expression of VEGFR-2 – such as in the thyroid and pancreatic islets – are sensitive for VEGF inhibition (Kamba et al. 2005). In myocardial infarction and sepsis, VEGFR-2 is a major regulator of vascular permeability and cardiac dysfunction (Weis et al. 2004; Yano et al. 2006).

The proliferative, permeability, and survival effects of VEGFR-2 signalling on endothelium may occur through different downstream cascades. The DNA synthesis and proliferative downstream effects of VEGFR-2 signalling are mainly mediated through the PLCγ-PKC-MAPK pathway (Shibuya 2003). In contrast, VEGF-induced vascular permeability may involve a transient dissociation of a complex between VEGFR-2, VE-cadherin and β-catenin – a process involving Scr activation (Weis et al. 2004). The endothelial survival effect of VEGFR-2 signalling involves at least the phosphatidylinositol 3’-kinase (PI3K)/Akt pathway (Gerber et al. 1998).

4.3.1.9. VEGFR-3

VEGFR-3 binds VEGF-C and VEGF-D, and it is a key regulator for lymphatic growth (Jeltsch et al. 1997; Karkkainen et al. 2004). VEGFR-3 signalling regulates cardiovascular development in embryos (Dumont et al. 1998), but later in the development and in adulthood it selectively regulates the growth and maintenance of lymphatic vessels (Makinen et al. 2001). In addition to lymphatic EC, VEGFR-3 is also expressed in macrophages and dendritic cells and regulates their migration (Hamrah et al. 2003; Chen et al. 2004b; Maruyama et al. 2005). Lymphatic vascular insufficiency leads to lymphedema (Irthum et al. 2000), and extensive lymphangiogenesis is often seen in inflammatory situations (Baluk et al. 2005) and tumor metastasis (Karkkainen et
al. 2002). VEGFR-3+ macrophages directly contribute to lymphangiogenesis by differentiating into lymphatic EC (Maruyama et al. 2005).

4.3.1.10. Neuropilins

The neuropilin receptors NRP1 and NRP2 are considered co-receptors for VEGF. They are expressed both in neuronal cells and EC and bind VEGF164 in addition to semaphorins (Eichmann et al. 2005). In the vascular system, NRP1 is expressed predominantly in arteries and potentiates the binding and activity of VEGF164 on VEGFR-2 (Soker et al. 1998). NRP2 is expressed and may modulate the function of veins and lymphatic vessels (Tammela et al. 2005; Karpanen et al. 2006). In addition to effects on the nervous and vascular systems, neuropilins may modulate immune functions. Dendritic cells express NRP1 (Tordjman et al. 2002) and the receptor may also be transferred to T cells by trogocytosis (Bourbie-Vaudaine et al. 2006). The transferred NRP1 on T cells can then bind VEGF possibly modulating EC functions in lymphoid organs (Bourbie-Vaudaine et al. 2006).

4.3.2. The angiopoietin family

The angiopoietin family currently consists of four ligands, the Ang1-4, and two receptors, the Tie1 and Tie2, that are primarily expressed by EC. Angiopoietins are important regulators of angiogenesis and vascular maintenance (Brindle et al. 2006; Eklund and Olsen 2006). The binding properties of angiopoietin ligands and the principal effects of Tie receptors are summarized in Figure 14.

4.3.2.1. Angiopoietin-1

Angiopoietin-1 binds both to Tie1 and Tie2 receptors although currently very little is known about Ang1/Tie1 signalling (Eklund and Olsen 2006). Ang1 is expressed mainly in mesenchymal cells such as SMC whereas the Tie2 is expressed in the endothelium indicating that Ang1 regulates the interaction of EC and surrounding supporting cells (Davis et al. 1996; Kim et al. 2000a; Kim et al. 2001a). Ang1-deficient mice die prenatally primarily due to defects of vascular remodelling and maturation similarly to the Tie2-deficient mice (Suri et al. 1996). Ang1 increases EC migration and tube formation but not EC proliferation. Ang1 has also anti-apoptotic, anti-permeability and anti-inflammatory effects on EC, and recruits pericytes and SMC to form support for newly-formed vessels (Eklund and Olsen 2006).

Ang1 reduces EC apoptosis initiated by different stimuli (Kwak et al. 1999; Kim et al. 2001a; Chen et al. 2004a) and this involves the activation of PI3K/Akt signalling, and ABIN-2 – an inhibitor of NF-kappa-β (Kim et al. 2000a; Tadros et al. 2003; DeBusk et al. 2004). In a monocrotaline-induced pulmonary hypertension model, Ang1 also has anti-apoptotic and protective effects on pulmonary endothelium, and hypertension (Zhao et al. 2003). Ang1 reduces vascular leakage in animals after VEGF administration or inflammation (Thurston et al. 1999; 2000; Visconti et al. 2002; Arsic et al. 2003; Baffert et al. 2006). The mechanisms behind the anti-permeability effects of Ang1 include reduction of the number of endothelial gaps in venules (Baffert et al. 2006), and increase in endothelial integrity (Gamble et al. 2000). Ang1 also inhibits TNF-α- or VEGF-induced tissue factor expression in EC (Kim et al. 2002), reduces VEGF-stimulated ICAM-1, VCAM-1 and E-selectin expression, impairs leukocyte adhesion to EC (Kim et al. 2001c), and activates ABIN-2 (Hughes et al. 2003; Tadros et al. 2003). Although Ang1 directly
induces eosinophil and neutrophil chemotaxis, it inhibits VEGF-induced migration of these cells (Feistritzer et al. 2004; Sturn et al. 2005). In a model for endotoxic shock, pretreatment with Ang1 improves cardiovascular function, vascular permeability, inflammation, and survival (Witzenbichler et al. 2005).

In addition to its endothelial effects, Ang1 indirectly regulates SMC functions, and has effects on progenitor cells. Stimulation of EC with Ang1 increases the production of potent SMC cytokines such as heparin-binding EGF-like growth factor (HB-EGF) (Iivanainen et al. 2003) and serotonin (Sullivan et al. 2003). Moreover, Ang1 overexpression in the lung results in pathological SMC proliferation and pulmonary hypertension (Sullivan et al. 2003; Chu et al. 2004). Ang1 mobilizes vascular and haematopoietic progenitor cells from the BM (Hattori et al. 2001) and has also receptor-independent effects as it induces cell attachment through integrins (Carlson et al. 2001) and enhances cardiomyocyte survival (Dallabrida et al. 2005). Taken together, Ang1 regulates the development and maintenance of the vasculature and has important protective effects on vascular permeability and inflammation.

*Figure 14.* Angiopoietin ligands and receptors. The effects of Ang1/Tie2 signalling are currently best characterized and include endothelial survival, vascular stabilization and maturation, haematopoiesis and vascular progenitor cell mobilization and recruitment. Ang2 is a context-dependent ligand that phosphorylates Tie2 and elicits downstream effects but may also antagonize the effects of Ang1. The role of Tie1 is poorly-known but may involve effects on vascular stability. Ang, angiopoietin; EC, endothelial cell; HSC, haematopoietic stem cell; SMC, smooth muscle cell.
4.3.2.2. Angiopoietin-2

Angiopoietin-2 binds to Tie2 but in contrast to Ang1, it is considered to be a context-dependent agonist/antagonist (Eklund and Olsen 2006). Ang2 is expressed in adults at sites of vascular remodelling (Maisonpierre et al. 1997). In contrast to Ang1, Ang2 is mainly expressed in the endothelium and is stored in Weibel-Palade bodies of EC (Fiedler et al. 2004). TNF-α induces Ang2 expression in EC (Kim et al. 2000c) whereas PDGF decreases Ang2 protein synthesis (Phelps et al. 2005). Transgenic Ang2 overexpression results in blood vessel disruption similar to the phenotype found in Ang1- or Tie2-deficient mice. This suggested that Ang2 is a naturally-occurring antagonist of Ang1/Tie2 signalling (Maisonpierre et al. 1997). Subsequent studies have shown that Ang2 may activate the Tie2 receptor by itself (Teichert-Kuliszewska et al. 2000). Accordingly, high Ang2 concentrations or its prolonged administration inhibits endothelial apoptosis (Kim et al. 2000b; Chen et al. 2004a).

Although Ang2 is not required for embryonal vascular development, it is required for vascular remodelling and lymphangiogenesis later in life (Gale et al. 2002). The effect of Ang2 on angiogenesis may depend on the availability other factors. Ang2 together with VEGF leads to ocular angiogenesis whereas it results in vascular regression in the absence of VEGF (Lobov et al. 2002). In advanced atherosclerotic plaques, Ang2 may inhibit plaque neovascularization by activating Tie2-mediated STAT5 signalling (Calvi et al. 2004). The effects of Ang2 on vascular permeability and inflammation may be context-dependent. By itself Ang2 induces vascular permeability but during inflammation it reduces cellular infiltration in tissues (Roviezzo et al. 2005). In contrast, a recent report shows that Ang2 reduces vascular permeability (Daly et al. 2006). In addition, Ang2 induces neutrophil migration but inhibits VEGF-induced neutrophil chemotaxis (Sturn et al. 2005). Similarly to Ang1, also Ang2 binds to integrins (Carlson et al. 2001) and mediates myocyte adhesion Tie2-independently (Dallabrida et al. 2005). In contrast to Ang1, Ang2 does not lead to expression of SMC mitogens from EC (Iivanainen et al. 2003). Ang2 has thus context-dependent effects on Tie2 signalling, vascular functions and inflammation, and it does not induce expression of SMC mitogens from the endothelium.

4.3.2.3. Tie1

Tie1 receptor is expressed in vascular endothelium but its functional role is currently poorly understood. Tie1-deficient mice die prenatally due to defects in vascular integrity indicating that Tie1 signalling is important in vascular quiescence (Eklund and Olsen 2006). Only recently it was found that Ang1 and Ang4, and particularly the angiopoietin derivate COMP-Ang1, induce phosphorylation of Tie1. In addition, formation of Tie1 and Tie2 heterodimers may modulate Tie1 signalling (Saharinen et al. 2005). Tie1 is expressed in EC that are under shear stress such as bifurcations (Porat et al. 2004).

4.3.2.4. Tie2

Tie2 is considered the principal receptor for angiopoietin effects, and it binds Ang1-4. Tie2 is expressed both in EC and HSC. Tie2-deficient mice die in utero due to impaired angiogenesis and cardiac development (Sato et al. 1995). Tie2 loss later in development leads to rapid EC apoptosis and blood vessel disruption showing an essential role for Tie2 in vascular maintenance (Jones et al. 2001b). Activating Tie2 mutations are encountered in venous malformations (Vikkula et al. 1996) and aberrant Tie2 signalling is linked also to intramuscular hemangiomas,
psorisis, pulmonary hypertension and tumours. The principal downstream pathway for Tie2 activation and its anti-apoptotic effects is PI-3K signalling (Eklund and Olsen 2006).

4.3.3. PDGF

Platelet-derived growth factors (PDGF) are potent mitogens for mesenchymal cells such as SMC and fibroblasts. PDGF was first found from platelets (Ross et al. 1974; Antoniades et al. 1979) and the growth factor family currently consists of four ligands (PDGF-A-D) that can form five different dimers. The ligand dimers have different binding affinity to PDGF tyrosine kinase receptors as shown in Figure 15. Studies with transgenic animal models suggest that PDGF-A and -C are the principal ligands for PDGFR-α (Ding et al. 2004) whereas PDGF-B and PDGF-D are the main ligands responsible for PDGFR-β activation (Betscholtz et al. 2004). PDGF-A and PDGF-B are secreted in activated forms whereas the more recently discovered novel PDGF members PDGF-C and PDGF-D need extracellular proteolytical processing (Fredriksson et al. 2004).

4.3.3.1. PDGF-A

During embryogenesis, PDGF-A is expressed in epithelial cells in close proximity to mesenchymal PDGFR-α expression suggesting paracrine signalling (Bostrom et al. 1996). In adults, PDGF-A is expressed most prominently in the heart, pancreas and skeletal muscle (Fredriksson et al. 2004). Low levels of PDGF-A in the adult cardiovascular system localize to SMC, platelets and monocytes/macrophages whereas the expression is induced in atherosclerosis (Evanko et al. 1998; Raines 2004). Increased blood pressure, sympathetic nerve activity, shear stress and hypercholesterolemia have been shown to increase PDGF-A expression (Raines 2004). The regulating transcription factors for PDGF-A expression include Egr-1 and Sp1 (Reigstad et al. 2005). In vitro, PDGF-AA is a weak stimulus for quiescent SMC but it induces strong SMC proliferation after inflammatory cytokine prestimulation and concomitant PDGFR-α upregulation (Sihvola et al. 1999). In arterial injury models, PDGF-AB antibody treatment that inhibits PDGF-AA, -AB and -BB decreases SMC accumulation in the intima (Ferns et al. 1991; Raines 2004).

4.3.3.2. PDGF-B

In the developing vasculature, PDGF-B is expressed in EC in close proximity to PDGFR-β expression in pericytes and SMC (Hellstrom et al. 1999). PDGF-B expression in human tissues is most prominent in heart and placenta (Fredriksson et al. 2004). In normal vasculature, low level PDGF-B expression localizes to SMC, monocyte/macrophages, B and T lymphocytes and platelets. In contrast, PDGF-B expression is upregulated in atherosclerotic arteries (Ross et al. 1990; Raines 2004). Shear stress and hypercholesterolemia induce PDGF-B expression in endothelium and circulating mononuclear cells (Raines 2004). In vitro, PDGF-BB induces strong SMC migration and proliferation (Sihvola et al. 1999) and also increases migration of monocytes and fibroblasts (Siegbahn et al. 1990). In vivo, PDGF-BB stabilizes blood vessels by recruiting supportive pericytes and SMC (Uutela et al. 2004) whereas loss of PDGF-B impairs recruitment of PDGFR-β+ pericytes and results in microhaemorrhages (Leveen et al. 1994; Lindahl et al. 1997). Exogenous PDGF-B increases SMC migration and accumulation to the intima of injured arteries (Jawien et al. 1992). In contrast, strategies that inhibit PDGF-B/PDGFR-β signalling have opposite effects (Leppanen et al. 2000; Raines 2004). PDGF-BB
also induces transdifferentiation of vascular progenitor cells to SMC (Yamashita et al. 2000; Simper et al. 2002).

**Figure 15.** PDGF ligands and receptors. PDGF ligands have different binding properties to PDGF receptors. During embryonic development, the principal ligands responsible for PDGFR-α signalling are PDGF-AA and -CC, and PDGF-BB and -DD induce PDGFR-β signalling. PDGF ligands are potent mitogens for cells of mesenchymal origin such as SMC and fibroblasts. In addition, especially the soluble PDGFR-β ligand PDGF-DD recruits inflammatory cells. SMC, smooth muscle cell.

### 4.3.3.3. PDGF-C

PDGF-C is expressed most prominently in heart, kidney and pancreas (Fredriksson et al. 2004). Unlike PDGF-A and PDGF-B, PDGF-C needs extracellular proteolysis for activation (Reigstad et al. 2005). PDGF-C expression is localized to endothelium, SMC, some monocyte/macrophages and platelets both in normal and atherosclerotic arteries (Uutela et al. 2001; Raines 2004). Egr1 and Sp1 transcription factors control PDGF-C expression (Reigstad et al. 2005). PDGF-CC induces SMC proliferation (Uutela et al. 2001), and PDGF-C overexpression in cardiomyocytes leads to VEGF upregulation, vascular permeability, SMC proliferation and cardiac fibrosis (Ponten et al. 2003). In postischemic heart and limb PDGF-CC induces revascularization, and recruits EC and SMC progenitors (Li et al. 2004).

### 4.3.3.4. PDGF-D

PDGF-DD binds only to PDGFR-ββ, and is proteolytically processed before activation (Bergsten et al. 2001). PDGF-D is mostly expressed in heart, pancreas and ovary (Fredriksson et al. 2004). PDGF-DD is a potent mitogen for SMC (Uutela et al. 2001). In vivo, PDGF-DD induces macrophage migration and recruits pericytes and SMC to blood vessels (Uutela et al. 2004). Overexpression of PDGF-D in cardiomyocytes leads to SMC proliferation and cardiac fibrosis (Ponten et al. 2005). In contrast, PDGF-D inhibition decreases fibrosis and scarring in
kidney glomerulonephritis (Ostendorf et al. 2006).

4.3.3.5. PDGFR-α

PDGFR-α is the principal receptor for PDGF-AA and PDGF-CC at least during embryonal development (Ding et al. 2004). In normal arteries, low PDGFR-α expression is found in SMC, lymphocytes and platelets. In atherosclerosis, PDGFR-α is induced in EC, SMC and monocyte/macrophages (Raines 2004). Pretreatment with inflammatory cytokines upregulates PDGFR-α expression in SMC and render them responsive for PDGF-AA proliferation (Sihvola et al. 1999). After arterial injury, PDGFR-α inhibition does not reduce intimal development (Giese et al. 1999).

4.3.3.6. PDGFR-β

PDGFR-β is the primary receptor for PDGF-BB and -DD and it is required for normal vascular formation. In developing blood vessels, paracrine PDGF-B expression in the endothelium drives the recruitment and proliferation of PDGFR-β+ pericytes and SMC that form support and stability for the vasculature (Betsholtz 2004). In normal adult arteries, only low PDGFR-β expression is detected in SMC and lymphocytes. In contrast, PDGFR-β expression is induced in EC, SMC, adventitial fibroblasts and monocyte/macrophages of atherosclerosis arteries (Raines 200). In vitro, quiescent SMC already have PDGFR-β expression rendering them readily responsive for PDGF-BB-induced migration and proliferation (Sihvola et al. 1999). In contrast to targeting PDGFR-α, PDGFR-β inhibition reduces intimal development after arterial injury (Giese et al. 1999).

4.4. Angiogenesis and vascular growth factors in heart transplantation

4.4.1. Angiogenesis in heart transplantation

In a skin transplantation model, significant angiogenesis is seen in the transplant after leukocyte infiltration, but before the destruction of the graft microvessels (Moulton et al. 1999). Angiogenesis is also seen in the intimal lesions of transplanted human hearts (Atkinson et al. 2005). In pediatric heart transplant patients, adventitial angiogenesis is associated with perivascular inflammation and correlates with the degree of TxCAD (Seipelt et al. 2005). Treatment with angiogenesis inhibitor TNP-470 reduced experimental TxCAD (Denton et al. 2004).

4.4.2. VEGF in heart transplantation

Both clinical and preclinical data are available on the role of VEGF in heart transplantation. It was first reported that VEGF immunoreactivity correlated with fibrin deposits, macrophage and neutrophil infiltrates and EC activation in cardiac allograft endomyocardial biopsies (Torry et al. 1995). Another study showed that VEGF expression in cardiac allograft biopsies is associated with infiltrations of T cells, monocytes and macrophages, all grades of acute rejection, and also indicates subsequent development of TxCAD (Reinders et al. 2003a). In paediatric heart transplant patients, VEGF serum levels are elevated during acute rejection, and decrease after immunosuppressive therapy (Abramson et al. 2002).
In experimental models, VEGF expression is associated with acute rejection (Reinders et al. 2003b). VEGF does not directly increase T cell proliferation, but it upregulates endothelial expression of adhesion proteins and potent leukocyte chemotactic cytokines (Reinders et al. 2003b). Anti-VEGF therapy prolongs cardiac allograft survival (Reinders et al. 2003b), and inhibition of both VEGFR-1 and -2 may be needed to inhibit strong acute rejection (Sho et al. 2005).

4.4.3. Angiopoietins in heart transplantation

No prior reports exist on the role of angiopoietins in transplanted organs.

4.4.4. PDGF in heart transplantation

Both clinical and preclinical data are available on the role of PDGF in arteriosclerosis after heart transplantation. In human cardiac allografts, the expression of PDGF ligands and receptors is increased (Zhao et al. 1994; Shaddy et al. 1996; Salom et al. 1998). Also, peripheral blood mononuclear cells from patients that later developed TxCAD preferentially show upregulated endothelial PDGF-A expression (Hosenpud et al. 1996). Experimental heart transplantation models indicate a clear correlation between PDGF ligand and receptor expression and the development of TxCAD (Lemstrom and Koskinen 1997). Further evidence on the regulatory role for PDGF on pathological SMC activation in cardiac allografts comes from studies where pharmacological PDGF inhibition effectively prevents the development of TxCAD (Sihvola et al. 1999; 2003). PDGF-A/PDGFR-α signalling may be important for the development of TxCAD (Lemstrom and Koskinen 1997; Sihvola et al. 1999; Mancini and Evans 2000) whereas PDGFR-β signalling seems to predominate in intimal growth in arterial injury models and atherosclerosis (Giese et al. 1999; Raines 2004).

5. Stem and progenitor cells of cardiac vasculature

Stem cells are undifferentiated cells that are capable of self-renewal and may differentiate to specialized cells. Progenitor cells and precursor cells are immature cells that have gained specialized features but lost their self-renewal capacity (Anversa et al. 2006). The existence of stem cells during embryonal development, and in haematopoiesis has been known for a long time. In contrast, the relatively recent finding of adult stem cells and progenitor cells, and their plasticity has boosted research for stem cell-based regenerative strategies including cardiac repair after myocardial infarction and in chronic heart failure (Anversa et al. 2006), and therapeutic angiogenesis (Boyle et al. 2006). In addition to their therapeutic potential, stem cells and progenitor cells are important in many pathological situations such as cancer (Burkert et al. 2006) and the development of neointimal lesions (Hillebrands et al. 2003). Stem cells and progenitor cells from various sources may participate in the maintenance and repair processes of vascular structures in adult heart (Figure 16). In the cardiac parenchyma, resident cardiac stem cells (CSC) can differentiate to EC and SMC lineages (Anversa et al. 2006). Adventitial cells that express stem cell markers can also transdifferentiate to vascular cells (Torsney et al. 2005). In addition to cells in the heart itself, extracardiac stem and progenitor cells may form vascular structures in the heart. These cells may originate from extracardiac vasculature (Torsney et al. 2005), BM (Aghi and Chiocca 2005) and possibly from other tissues with resident stem cells.
5.1. Resident cardiac stem cells

The heart itself harbours a pool of resident cardiac stem cells (CSC) that are considered to participate in the continuous turnover of cardiac cells. These cells are abundant after birth, decrease gradually during the early post-natal period and reside preferably in stem cell niches in the atrium and apex (Quaini et al. 2002; Anversa et al. 2006; Gude et al. 2006). Different cell surface and functional properties have been described for CSC. These cells do not have haematopoietic markers but express stem cell antigens c-kit, sca-1 and MDR1 (Figure 17) (Beltrami et al. 2003; Oh et al. 2003; Anversa et al. 2006). In addition, CSC have the ability to efflux Hoechst dye (property of side population cells, SP) (Hierlihy et al. 2002).

CSC may divide to replenish the stem cell pool. In addition, the CSC differentiate to lineage-committed progenitor cells, precursor cells, transiently-dividing cells, and finally to EC, SMC or cardiomyocytes (Figure 17) (Anversa et al. 2006). Insulin-like growth factor-1 mediates CSC survival and proliferation, whereas HGF induces matrix metalloproteinase (MMP)-2/9-dependent CSC migration (Linke et al. 2005). Also, nuclear Akt signalling is important in the CSC cycling (Gude et al. 2006). In normal conditions, CSC preferably retain their immature phenotype (Steele et al. 2005) whereas myocardial infarction in adults increases the number of CSC (Urbanek et al. 2005) and may promote their endothelial differentiation (Steele et al. 2005). During ischemic insult, BM-derived stem cells contribute to the CSC pool (Mouquet et al. 2005). In transplanted human hearts, the number of CSC in the ventricle is increased and differentiation to EC, SMC and cardiomyocytes has been observed. A fraction of these primitive cells originate from the recipient whereas the majority are derived from the donor (Quaini et al. 2002).
Figure 17. Differentiation of cardiac stem cells. Stem cells such as cardiac stem cells normally reside in a quiescent state, and are capable of self renewal and differentiation to lineage-committed cells and finally to fully differentiated cells. During the differentiation process, lineage-specific transcription factors are activated (progenitors and precursors) whereafter lineage-specific proteins are expressed (precursors). The stem cell antigens c-kit, Sca-1 and MDR-1 are lost during the amplifying phase of the cells before they fully maturate. EC, endothelial cell; αSMA, α-smooth muscle actin; SMC, smooth muscle cell. Modified from Anversa et al. 2006.

5.2. Adventitial stem and progenitor cells

Adventitia of mouse aorta contains cells that express stem cell and progenitor markers such as c-kit, Sca-1, CD34 and VEGFR-2 (Hu et al. 2004), or are SP cells (Sainz et al. 2005). Adventitial stem cells may differentiate to SMC after PDGF-BB stimulus, and participate in neointimal formation of vein grafts (Hu et al. 2004). Adventitial SP progenitor cells have been shown to differentiate to EC and SMC by VEGF and PDGF, respectively (Sainz et al. 2005). In addition to mouse tissues, adventitial cells that express stem cell markers have also been found in human arteries (Torsney et al. 2006).

5.3. Circulating endothelial cell and smooth muscle cell progenitors

In addition to erythrocytes, leukocytes and platelets, adult circulation contains cells that can differentiate to EC or SMC. The origin of these cells is probably heterogeneous as both BM and non-BM-derived cells may differentiate to vascular structures (Hillebrands et al. 2002). EPC were first described as VEGFR-3+CD34+ mononuclear cells (Asahara et al. 1997). They also express CD133 and CXCR4 and migrate in response to VEGF and stromal-derived factor...
(SDF)-1 (Peichev et al. 2000). In addition, CD14+CD34- myeloid cells (Urbich et al. 2003) and AC133-CD14+ myelomonocyte cells may differentiate to endothelial cells (Kim et al. 2005) indicating that EPC is a heterogeneous cell population. In vitro, EPC can be differentiated to EC by VEGF (Urbich and Dimmeler 2004). EPC are mobilized to peripheral circulation after myocardial infarction (Shintani et al. 2001; Massa et al. 2005). The mobilization involves VEGF, Ang1, granulocyte colony-stimulating factor (G-CSF), granulocyte-monocyte colony-stimulating factor (GM-CSF) and SDF-1, whereas hypoxia, inflammation, cytokines VEGF, MCP-1 and SDF-1, and integrins are potent signals for progenitor cell recruitment to the target tissues (Urbich and Dimmeler 2004; Dammeler et al. 2005). Although EPC may transdifferentiate to EC in vivo, paracrine effects of these cells may be more important in neovascularization (Urbich et al. 2005; Grunewald et al. 2006; Zentilin et al. 2006). The number of circulating EPC is lower in heart transplant patients with TxCAD than without transplant arteriosclerosis. In addition, recipient-derived EC were detected in the lesions indicating a possible role for EPC in TxCAD (Simper et al. 2003). Human circulating SMC progenitor cells have been described and these cells differentiate to SMC by PDGF-BB (Simper et al. 2002).

5.4. Bone marrow-derived vascular stem and progenitor cells

Several cells in the adult BM participate in the development of vascular cells (Urbich and Dimmeler 2004) and have been used in clinical studies aiming for cardiac repair (Boyle et al. 2006). BM contains EPC that have potential for vascular differentiation as discussed above. In addition, BM-derived mesenchymal stem cells can differentiate to EPC and further to EC (Reyes et al. 2002). In some animal models, BM-derived cells participate in physiological and pathological postnatal neovascularization (Asahara et al. 1999; Espinosa-Heidmann et al. 2005). In other models, BM-derived cells do not differentiate to EC during angiogenesis (Ziegelhoeffer et al. 2004) but may be important mediators for paracrine angiogenic signals (Fazel et al. 2006; Grunewald et al. 2006). In favour of a paracrine role for BM-derived cells in angiogenesis, BM-derived cells recruited to tissue by VEGF and SDF-1 secrete potent angiogenic factors inducing the proliferation of resident endothelium while the differentiation of BM cells to EC was minimal (Grunewald et al. 2006).

6. Novel therapeutic tools for vascular biology and heart transplantation

Studies aiming at either inhibiting tumour angiogenesis, or inducing therapeutic vascular growth in ischemic diseases have resulted in novel tools that may potentially be used in heart transplant recipients. These tools include gene vectors, receptor tyrosine kinase (RTK) inhibitors and monoclonal antibodies.

6.1. Gene vectors

Gene vectors can be used to introduce potentially therapeutic genes to somatic cells. Several clinical trials have been initiated since the early 1990’s to investigate the therapeutic potential of gene therapy (Gene Therapy Clinical Trials Worldwide, www.wiley.co.uk/genmed/clinical, The Journal of Gene Medicine). Despite some successful trials on monogenic diseases (Cavazzana-Calvo et al. 2000) and cancer (Immonen et al. 2004), attempts to induce therapeutic vascular growth in coronary heart disease or peripheral vascular disease have yielded mixed results (Yla-Herttuala and Alitalo 2003). Gene vectors have been generally well-tolerated but safety issues were raised after the death of a patient after intraportal injection of adenovirus vector
(Marshall 1999), and retrovirus induced insertional mutagenesis in two paediatric patients (Hacein-Bey-Abina et al. 2003).

Both non-viral and viral vectors have been used as gene vectors (Gene Therapy Clinical Trials Worldwide). Non-viral vectors include plasmid DNA and cationic liposomes that are well-tolerated but inefficient compared to viral vectors (Thomas et al. 2003). Adenoviruses (Ad) and retroviruses are the most commonly-used viral vectors but adeno-associated viruses (AAV), pox viruses and herpes simplex virus -1 have also been used (Gene Therapy Clinical Trials Worldwide). Utilization of viruses as gene vectors generally includes partial deletion of the viral genome and insertion of the gene of interest. After receptor-mediated endocytosis, the vector genome is transferred to the target cell nucleus, and the transcription of the gene is initiated. Different viral vectors vary in their transfection efficiency, tropism for target cells, gene packaging capacity, host inflammatory response, stability of the transgene expression, ability of infect dividing or non-dividing cells, and whether the transgene integrates to the host genome (Thomas et al. 2003).

Heart transplantation is a good candidate for gene therapy because gene vectors may be easily applied during the operative ex vivo time of the transplanted organ. Adenovirus (Wang et al. 1996; Kypson et al. 1998), lentivirus (Zhao et al. 2005), AAV (Svensson et al. 1999) and liposome vectors (Furukawa et al. 2005) have been used to achieve transeGene expression in transplanted hearts in preclinical models. Therapeutic efforts include strategies targeting co-stimulation, cell adhesion, protective genes or pathological SMC proliferation (Isobe et al. 200). One option to use gene therapy in transplantation is pretransplant donor MHC gene transfer to achieve tolerance. In clinical practice, transferring only one MHC gene with high prevalence, and not expressed by the recipient could be sufficient to induce tolerance through linked suppression (Wong and Wood 2004).

6.2. Protein tyrosine kinase inhibitors and monoclonal antibodies

Numerous pharmacological agents targeting tumour vasculature are currently being developed. These include monoclonal antibodies and small-molecule RTK inhibitors. Monoclonal antibodies may specifically bind to the ligand or to the receptor. Monoclonal anti-VEGF antibody bevacizumab has shown efficacy in colorectal (Hurwitz et al. 2004), lung and breast cancers and is approved for clinical use (Jain et al. 2006).

Protein kinases are enzymes that attach phosphate groups to serine, threonine and/or tyrosine residues, thereby altering the functional properties of the target molecules. RTK inhibitors are potent small molecular drugs with selective binding to the ATP site of the receptors (Force et al. 2004). Imatinib is a potent PDGFR inhibitor and inhibits also Bcr-Abl and c-kit but not other receptor or cytoplasmic tyrosine kinases. Imatinib has been used with success to treat chronic myelogenous leukemia where Bcr-Abl fusion protein is generated and active. In addition, imatinib is in clinical use for treating gastrointestinal stromal tumor (GIST) – a disease with aberrant c-kit signalling (Buchdunger et al. 2002). PTK787, on the other hand, is a potent VEGFR inhibitor and can inhibit also other class III kinases, such as PDGFR-β tyrosine kinase, c-kit and c-Fms, but at higher concentrations. PTK787 is currently in phase III cancer studies (Wood et al. 2000; Hess-Stumpp et al. 2005; Jain et al. 2006).
AIMS OF THE STUDY

The aim of this study was to investigate the role of angiogenesis and vascular growth factors in the development of experimental TxCAD. Also, this study aimed to identify potential therapeutic targets by pharmacological and gene therapy approaches.

The specific aims of this study were:

1. to investigate angiogenesis and the origin of endothelial cells in transplanted hearts;
2. to investigate the expression and role of vascular growth factors during alloimmune responses in transplanted hearts;
3. to target vascular growth factors with novel pharmacological approaches in cardiac allograft arteriosclerosis; and
4. to target vascular growth factors with novel gene therapy approaches in cardiac allograft arteriosclerosis.
METHODS

1. Heterotopic heart transplantation models

Rabbit, rat and mouse heterotopic heart transplantation models were used (Table 4). Suboptimal immunosuppression was applied to prevent severe acute rejections and to achieve the development of arteriosclerotic changes. In the rabbit model, the recipients were also fed with a cholesterol-rich diet. Permission for animal experiments was obtained from the State Provincial Office of Southern Finland. The animals received care in compliance with the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Academy Press (ISBN 0-309-05377-3, revised 1996).

Table 4. Chronic rejection heart transplantation models

<table>
<thead>
<tr>
<th>Animal</th>
<th>Donor</th>
<th>Recipient</th>
<th>Position</th>
<th>Anesthesia</th>
<th>Immunosuppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>C57</td>
<td>Balb</td>
<td>Abdominal</td>
<td>Fentanyl/fluaniison/buprenorfine</td>
<td>FK506</td>
</tr>
<tr>
<td>Rat</td>
<td>DA</td>
<td>WF</td>
<td>Abdominal</td>
<td>Isoflurane/buprenorfine</td>
<td>CsA</td>
</tr>
<tr>
<td>Rabbit</td>
<td>DB</td>
<td>NZB</td>
<td>Cervical</td>
<td>Ketamin/medetomin/buprenorfine</td>
<td>CsA</td>
</tr>
</tbody>
</table>

CsA, Cyclosporine A; DA, Dark Agouti; DB, Dutch Belted; IS, immunosuppression; NZW, New Zealand White; WF, Wistar Furth.

Rat heart transplantation. Specific pathogen-free inbred male Dark Agouti (DA, RT1av1) and Wistar Furth (WF, RT1u) rats weighting 250-300 g (Harlan, Horst, The Netherlands) and of 2-3 months of age were used. Heterotopic cardiac transplantations were performed from DA to DA rats (syngrafts) or from DA to WF strains (allografts, fully MHC–mismatch). Isoflurane (Isofluuran Baxter, Deerfield, IL) was used for anaesthesia and buprenorphine (Temgesic, Schering-Plough, Kenilworth, NJ) at a dose of 0.1 mg/kg for operative and post-operative analgesia.

During the operation, the donor heart was first perfused with 10 ml of +4°C PBS with 500 IU heparin through the vena cava inferior. The caval vessels and pulmonary veins were ligated and the aorta and pulmonary artery were cut 3-5 mm above their origin. The donor heart was preserved in +4°C PBS for 15 min during which time the abdominal aorta and inferior vena cava of the recipient were prepared, and clamped between the renal vessels and bifurcation. The aorta and pulmonary vein of the donor heart were next anastomosed end-to-side to the recipient abdominal aorta and inferior vena cava, respectively, using microsurgical techniques modified from Ono and Lindsey (Ono and Lindsey 1969). The donor heart was kept cold during the anastomosis by applying +4°C PBS. The total ischemic time was 45±15 min. The clamps were then opened and the graft again gained pulsatile activity upon reperfusion.

In the acute rejection model, the recipients were left non-immunosuppressed and the allografts were harvested five days after the transplantation. In the chronic rejection model, recipients received suboptimal CsA (see drug regimens) immunosuppression. The allografts were harvested eight weeks after the transplantation or if the function of the heart deteriorated, determined by daily palpation. In characterization studies, non-transplanted hearts and syngrafts were used as controls.

Mouse heart transplantation. Specific pathogen-free inbred male Balb/c (B/c, H-2d) and C57BL/6J (B6, H-2b) mice weighing 25-30 g (Harlan) were used. Heterotopic cardiac allografts were transplanted from Balb/c to C57 mice (fully MHC–mismatch) to the abdominal position
applying similar microsurgical techniques as with the rat transplantations. A combination of fentanyl/fluanisone (Hypnorm) and midazolam (Dormicum) was used for anesthesia and analgesia, and 0.1 mg/kg of buprenorphine for post-operative analgesia. The recipients were treated with suboptimal FK506 background immunosuppression (see drug regimens) to achieve chronic rejection changes, and the allografts were harvested eight weeks after the transplantation.

**Rabbit heart transplantation.** Specific pathogen-free outbred female Dutch Belted (DB, 1.5-2 kg) and New Zealand White (NZW, 2-3.5 kg) rabbit strains (Harlan) were used. Heterotopic heart transplantations were performed into cervical position from DB to NZW rabbits. Donor hearts were harvested under ketamine 20 mg/kg, and medetomidin 300 mg/kg intramuscular anesthesia. Intravenous heparin and pentobarbital 30 mg/kg was administered to the donors just before cardiectomy. The donor heart was harvested as described in the rat heart transplantation model. Recipients were anesthetized using intramuscular ketamine 20 mg/kg, and medetomidin 300 mg/kg. Subcutaneous buprenorfine 0.04 mg/kg was given for analgesia. Before operation, recipients were given intravenous kefalotin (Keflin, Eli Lilly Ltd., Vantaa, Finland) as prophylactic antibiotics and atropin for hemodynamic stability.

After harvesting, the aorta and pulmonary vein of donor heart were anastomosed end-to-side to the common carotid artery and jugular vein of the recipient, respectively. The donor heart was kept cold during the anastomosis by applying +4°C PBS and the total ischemic time was 70±20 min. To induce arteriosclerotic changes, the allograft recipients received suboptimal CsA immunosuppression (see drug regimens) and were fed with a 0.5 % cholesterol diet (Altromin International, Lage, Germany) (Alonso et al. 1977) starting four days before the transplantation. The allografts were harvested 30 days after transplantation.

2. Drug regimens

**Cyclosporine A.** Cyclosporine A (CsA, Novartis, Basel, Switzerland) was used as background immunosuppression. It was diluted in Intralipid (Fresenius Kabi, Bad Homburg, Germany). In the rat chronic rejection model, cardiac allograft recipients received 2.0 mg/kg/d of CsA subcutaneously for the first week and 1.0 mg/kg/d thereafter. Whole-blood CsA 24-hour through levels were determined weekly by radioimmunoassay (Sandimmun-Kit, Novartis). In the rabbit chronic rejection model, the recipients received 3.0 mg/kg/d of CsA subcutaneously.

**FK506.** FK506 (tacrolimus; i.m. formulation, Astellas Pharma, Tokyo, Japan) was used as background immunosuppression. It was diluted in 0.9% NaCl. In the mouse chronic rejection model, the recipients received 3.0 mg/kg/d of FK506 subcutaneously for the first week and 1.5 mg/kg/d thereafter.

**PTK787.** Receptor tyrosine kinase inhibitor PTK787 (vatalanib, PTK/ZK, PTK787/ZK222584, Novartis) was used as it inhibits VEGFR-signalling. It was diluted in polyethylene glycol and given at 100 mg/kg/d perorally b.i.d. PTK787 is a potent VEGFR inhibitor and can also inhibit other class III kinases, such as PDGFR-β tyrosine kinase, c-Kit, and c-Fms, but at higher concentrations (Wood et al. 2000; Hess-Stumpp et al. 2005).

**Anti-VEGFR-antibodies.** Neutralizing anti-mouse VEGFR-1 (MF1, ImClone, New York, NY) and -2 antibodies (DC101, ImClone) were used to specifically inhibit the respective VEGFR-
signalling. 800 µg of the respective antibody was administered intraperitoneally every third day for 30 days.

**Imatinib.** Receptor tyrosine kinase inhibitor imatinib (STI571, Novartis) was used as it inhibits PDGFR-signalling. It was diluted in saline and administered at 10 mg/kg per day intraperitoneally. Imatinib is a potent PDGFR inhibitor and inhibits also Bcr-Abl and c-kit but not other receptor or cytoplasmic tyrosine kinases (Buchdunger *et al.* 2002).

### 3. Gene transfer

After harvesting, cardiac allografts were perfused intracoronary with adenovirus vectors or adeno-associated virus (AAV) vectors to achieve transgene expression in the transplanted hearts. Concomitantly with the gene vector, 50 µL of 10^7 mol/L acetylcholine was used to permeabilize the endothelium and enhance transfection efficiency. After vector perfusion, the aorta and the pulmonary artery of the heart transplant were clamped and the heart was preserved in +4°C PBS for 15 min before proceeding with the operation.

**Adenovirus vectors.** Adenovirus vectors (serotype 5) were obtained from Professor Seppo Ylä-Herttuala, University of Kuopio. Replication-deficient E1-E3–deleted clinical GMP-grade adenoviruses (serotype 5) were produced in 293T cells. Adenoviruses were analyzed to be free of replication-competent viruses, lipopolysaccharide, mycoplasma, and other microbiological contaminants. Cardiac allografts were perfused with adenoviruses that express β-galactosidase (Ad.LacZ, control), mouse VEGF_{164} (Ad.VEGF) or human Ang1 (Ad.Ang1) under CMV promoter. 1x10^9 and 2x10^8 plaque-forming units were used in the rabbit and rat transplantation models, respectively.

**AAV vectors.** AAV vectors (serotype 2) were obtained from Docent Katri Pajusola and Professor Kari Alitalo, University of Helsinki. AAV vectors were produced in 293T cells and purified by an iodixanol-gradient ultracentrifugation and heparin-Sepharose high-performance liquid chromatography. Cardiac allografts were perfused with AAV vectors that express enhanced green fluorescent protein (AAV.EGFP, control), luciferase (AAV-Luc, control), human Ang1 (AAV-Ang1) or human Ang2 (AAV-Ang2) under CAG (AAV-EGFP) or CMV promoter (AAV-Ang1 and AAV-Ang2). 2x10^{11} genomic particles (AAV-Ang1, AAV-Ang2) or 1.4x10^{11} genomic particles (AAV-Luc) were used.

**Detection of transgene expression.** X-gal staining was used to reveal Ad.LacZ-mediated transgene expression. Anti-EGFP-antibodies were used to reveal AAV-EGFP-mediated transgene expression. Bioluminescent imaging (IVIS, Xenogen, Alameda, CA) was used to non-invasively reveal AAV-Luc-mediated transgene expression. Recipients were anesthetized and received the luciferase substrate D-Luciferin (bc29, SynChem OHG, Kalles, Germany) 150 mg/kg intraperitoneally 10 minutes before bioluminescent imaging. AAV-Ang1 and AAV-Ang2 transgene expression was confirmed with PCR using a construct specific forward primer and backward primers specific for Ang1 and Ang2, respectively.

### 4. Reporter gene animals

Transgenic reporter gene animals (Tie1/LacZ and GFP-BM) were used as cardiac allograft recipients to study the origin of cells in the transplanted hearts.
**Tie1/LacZ.** Wistar rats that express LacZ under the promoter of EC receptor Tie1 (Tie1/LacZ) were used (Porat et al. 2004). To investigate donor-derived Tie1 expression in transplanted hearts, Tie1/LacZ hearts were transplanted to non-immunosuppressed wild type recipients. To investigate the replacement of EC with recipient-derived Tie1-expressing cells in transplanted hearts, DA allografts were transplanted to Tie1/LacZ recipients. Reporter gene expression was revealed with X-gal staining.

**GFP-BM.** Replacement of allograft cells with BM-derived cells was investigated using C57 mice that had received a BM transplant from green fluorescent protein-expressing syngenic mice (GFP-BM) as Balb cardiac allograft recipients (Rajantie et al. 2004). Reporter gene expression was readily detected with a fluorescent microscope from samples that were incubated after harvesting in 20% sucrose overnight.

### 5. Histological evaluation

Cardiac allograft arteriosclerosis was determined in a blinded manner from paraformaldehyde-fixed paraffin sections stained with hematoxylin-eosin and Resorcin fuchsin for internal elastic lamina. The basal mid-section of the heart was used in histological evaluation. TxCAD was evaluated using either Billingham’s criteria (Lurie et al. 1981) or using computer-assisted morphometry. The Billingham’s criteria may be more sensitive to moderate intimal changes. In addition to the intensity of intimal development, the incidence of affected arteries was determined. All analyses were performed by two independent observers.

**Billingham’s criteria.** The arteries were graded as follows: grade 0, normal artery with intact internal elastic lamina; grade 1, <10% occlusion of lumen by arterial intimal thickening and proliferation, disruption of internal elastic lamina, some foam or vacuolated endothelial cells may be present; grade 2; <50% occlusion of the lumen; grade 3, >50% but <100% occlusion of lumen; and grade 4, 100% vessel occlusion of lumen.

**Computer-assisted morphometry.** Image processing software (NIH Image version 1.62, US National Institutes of Health; http://rsb.info.nih.gov/nih-image/) was used to determine the area surrounded by the internal elastic lamina, and the vessel lumen. Arterial occlusion percentage was determined as the ratio of neointimal area and internal elastic lamina area.

### 6. Immunohistochemistry and immunofluorescence stainings

Immunohistochemistry and immunofluorescence stainings were performed using frozen sections. The apical mid-section of the heart was embedded in TissueTek and snap-frozen in liquid nitrogen. Frozen heart sections (4 μm) were air-dried on silane-coated slides, and fixed in acetone for 20 min. After incubation with appropriate 1.5% nonimmune serum, sections were incubated with primary antibodies, and either immunohistochemical or immunofluorescent detection was used as described below. The antibodies and dilutions used in the study are listed in Table 5. Monoclonal antibodies were incubated at room temperature for 30 min and polyclonal antibodies at +4°C overnight. Specificity controls were performed by omitting the primary antibody, using the same immunoglobulin concentration of species- and isotype-matched antibodies, or preincubating the primary antibodies with excess of the corresponding blocking peptide when available.
**Immunohistochemical stainings.** Peroxidase ABC method (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA) and 3-aminono-9-ethylcarbazole (Vectastain) were used. After incubation with the primary antibody, the samples were incubated with biotinylated secondary antibodies for 30 min, followed by avidin-biotinylated horse-radish complex for 30 min, and finally with AEC containing 0.1% hydrogen peroxidase to yield a brown-red reaction product. The specimens were counterstained with hematoxylin and coverslips were mounted (Aquamount, BDH Ltd., Poole, UK).

**Immunofluorescent stainings.** Immunofluorescence double stainings were performed in a sequential manner. After primary antibody, the samples were incubated with Alexa Fluor 488 (green) or 568 (red, Promega, Madison, WI) secondary antibodies for 30 min. The specimens were mounted with Vectashield Mounting Media with DAPI (Vector Laboratories).

**Tyramine signal amplification.** LYVE-1 and MHC I stainings were performed with tyramine signal amplification kit (TSA, Perkin Elmer, Wellesley, MA) according to the Manufacturer’s instructions.

**Analysis of immunohistochemical stainings.** The stainings were analyzed in a blinded manner by two independent observers by counting positive cells or by grading the intensity of the staining, depending on the immunoreactivity pattern of the respective primary antibody. Clearly identifiable and quantifiable cells such as parenchymal inflammatory cells were counted from four random fields from each quadrant of the graft section using 400x magnification and the results are given as the mean density of positive cells. A more diffusible staining pattern such as VEGF immunoreactivity was graded from 0 to 3 as follows: 0, no visible expression; 1, few cells with faint expression; 2, moderate intensity with multifocal expression; and 3, intense expression.

**Fluorescent-activated cell sorting (FACS).** FACS was performed using a lyse-and-wash procedure. Peripheral blood was first incubated with the FITC-conjugated antibody for 15 min after which the erythrocytes were lysed with Cellkit C-04 (Cellset, Galmiz, Switzerland). 10 000 cells were then analyzed with a FACScan (Becton Dickinson, Franklin Lakes, NJ) flow cytometer. Peripheral blood leukocyte count was determined with Coulter T890 (Beckman Coulter, Fullerton, CA) cell counter, and the absolute circulating cell numbers were determined by multiplying the leukocyte count by the percentage of positive cells determined by FACS. The antibodies used are listed in Table 5.

7. **RNA assays**

**In situ hybridization.** Chromogenic and radioactive in situ hybridizations were performed. Paraffin sections were used in both cases. Chromogenic in situ hybridization was done with the Ventana Discovery (Ventana Medical Systems, Tucson, AR) automate. DIG-labeled antisense or sense probes were hybridized after deparaffinization, post-fixation, RiboClear pre-treatment and proteinase treatment. Samples were then incubated with biotinylated anti-DIG antibody (Jackson ImmunoResearch Laboratory, West Grove, PA) followed by BCIP/NBT substrate for color reaction. The sections were counterstained with Nuclear Fast Red (Merck, Darmstadt, Germany), dehydrated, and mounted with Mountex (HistoLab, Göteborg, Sweden). For radioactive in situ hybridization, radiolabeled antisense and sense RNA probes were synthesized with [35S]UTP (Amersham, Arlington Heights, IL). Paraffin sections were deparaffinized,
### Table 5. Antibodies used in the study

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<tr>
<th>Antibody</th>
<th>Clone / catalogue number</th>
<th>Dilution</th>
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<td>VEGFR-2</td>
<td>sc-315P</td>
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<td>Santa Cruz</td>
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treated in prehybridization solutions and probes were hybridized overnight. After high stringency washes, the sections were dehydrated, air-dried, dipped into NTB-2 emulsion (Eastman Kodak, Rochester, NY), and exposed at +4°C for 4 to 6 weeks. The sections were developed in D19 (Eastman Kodak), fixed in sodium fixative (Eastman Kodak), and counterstained with Mayer’s hemalum (Shandon, Pittsburgh, PA).

The probes used were human VEGF (X62568, bp 57–639, in pGEM3z, Promega), human VEGFR-1 (NM00209, bp 1–1898, in pGEM3z), human VEGFR-2 (X61656, bp 1–714, in pBluescript II, Stratagene, La Jolla, CA), rat PDGF-A (L06894, bp 305–780, pBluescript II), rat PDGF-B (Z14117, bp 149–528, pBluescript II), rat PDGFR-α (M63837, bp 124–687, pGEM4z), and rat PDGFR-β (Z14119, bp 15–425, pGEM4z). VEGF, VEGFR-1, and VEGFR-2 templates were a kind gift from Professor Kari Alitalo (Molecular Cancer Biology Laboratory, University of Helsinki).

**RNA isolation and reverse transcription.** Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription of mRNA was performed using M-MLV reverse transcriptase (Sigma-Aldrich, St. Louis, MO), recombinant RNasin ribonuclease inhibitor (Promega) and random nonamers (Sigma-Aldrich).

**Real time RT-PCR.** Real-time RT-PCR reactions were carried out in a LightCycler using LightCycler FastStart DNA MasterPLUS SYBR Green I mix (Roche, Basel, Switzerland). Measurement of the PCR product was performed at the end of each extension period. The number of mRNA copies of the gene of interest was calculated from a corresponding standard curve using LightCycler software (Roche) and given in relation to 18S rRNA or β-actin mRNA molecule numbers.

**8. Cell culture assays**

To study the effect of PDGF-AA, -BB, VEGF-A, and imatinib and PTK787 on the functional activity of SMC in vitro, migration and proliferation assays with rat coronary artery SMC (Dariusz Leszczynski; Finnish Centre for Radiation and Nuclear Safety, Helsinki, Finland) were used. In the assays, either no growth factors, human recombinant PDGF-AA (Sigma) 50 ng/mL, PDGF-BB (Sigma) 50 ng/mL, or VEGF (Upstate, Lake Placid, NY) 50 ng/mL were used. Also, PTK787 (1 µM), imatinib (1 µM) or both PTK787 and imatinib were added to each growth factor setting

**Smooth muscle cell migration assay.** A modified Boyden chamber method was used. 50 000 rat coronary artery SMC were added to the upper well of collagen-coated chambers. Media with 0.5% FCS and 0.1% bovine serum albumin was added in the lower well of the chamber. Also, the growth factors and tyrosine kinase inhibitors described above were added to the lower wells. After 24 hours of incubation, the cells that had migrated to the lower well were fixed with methanol, stained with Mayer’s hemalum, and 10 random fields with x20 magnification were counted microscopically.

**Smooth muscle cell proliferation assay.** 5000 SMC were seeded on 96-well culture plates in DMEM (GibCo, Invitrogen, Carlsbad, California) with 10% fetal calf serum (FCS, GibCo), glutamine, penicillin, and streptomycin. After 24 hours, the cells were starved for 72 hours in serum free media. Cell proliferative activity was determined 24 hours after the addition of growth
factors and drugs by using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium) assay (Celltiter 96, Promega). The experiments were repeated in triplicate.

9. Statistical methods

All data are expressed as mean ± SEM and analysed using Statview 512+ (Brain Power Inc., Calabasas, CA) or SPSS 11.5.1 (SPSS Inc, Chicago, IL) software. In non-parametric comparisons, Mann-Whitney test was used for two group comparisons and Kruskal-Wallis test with Dunn correction was used for multiple group comparisons. In parametric comparisons, Student t test was used for two group comparisons, ANOVA-test with Bonferroni correction to compare all groups and with Dunnett´s correction to compare the treatment groups to the control group. Linear regression analysis was applied to evaluate the correlation. P<0.05 was regarded as statistically significant.
RESULTS

1. Heterotopic heart transplantation models (I-V, and unpublished results)

Rodent heterotopic heart transplantation models were used to study the role of vascular growth factors and progenitor cells during acute rejection and the development of TxCAD. To investigate the effect of acute rejection (I, II and IV), fully MHC-mismatched rat cardiac allograft recipients were left without immunosuppression and the grafts were removed 5 days after transplantation. At this time points, the contractile function was still preserved but intense mononuclear cell infiltration was seen throughout the allograft as shown in Figure 18. In contrast, syngeneic controls were devoid of inflammatory infiltrates.

Heart transplantation models of chronic rejection were used to study the development of TxCAD. Using our previously-characterized rat model of chronic rejection (Sihvola et al. 1999), treatment with suboptimal CsA immunosuppression resulted in 75-100% long-term allograft survival together with the development of moderate inflammation, fibrosis and TxCAD (Figure 18, I-V). Only minimal chronic rejection changes were seen in similarly-treated syngeneic controls.
A rabbit chronic rejection model was developed to utilize gene therapy and to include a cholesterol diet that predisposes to the development of TxCAD. Suboptimal immunosuppression together with the cholesterol diet resulted in moderate chronic inflammation and the development of TxCAD 30 days after transplantation (I).

Table 6. Effect of different FK506 dosing in mouse cardiac allografts.

<table>
<thead>
<tr>
<th>Group</th>
<th>FK506 (mg/kg/d)</th>
<th>n=</th>
<th>End-point</th>
<th>Allograft survival</th>
<th>Intimal development (%)</th>
<th>Fibrosis (0-3)</th>
<th>Note</th>
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<td>1</td>
<td>0.3</td>
<td>13</td>
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<td>12</td>
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</tr>
<tr>
<td>3</td>
<td>3.0</td>
<td>6</td>
<td>6 weeks</td>
<td>50 %</td>
<td>17 %</td>
<td>66 %</td>
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</tr>
<tr>
<td>4</td>
<td>3.0</td>
<td>7</td>
<td>6 weeks</td>
<td>86 %</td>
<td>12 %</td>
<td>63 %</td>
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</tr>
<tr>
<td>5</td>
<td>3.0</td>
<td>7</td>
<td>6 weeks</td>
<td>100 %</td>
<td>11 %</td>
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</tr>
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<td>8</td>
<td>8 weeks</td>
<td>25 %</td>
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<td>2.8</td>
<td>6/8 grafts destroyed</td>
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<td>8</td>
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<td>88 %</td>
<td>56 %</td>
<td>83 %</td>
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</table>

In addition, a mouse model of chronic rejection with FK506 background immunosuppression was developed to allow the use transgenic mice and monoclonal neutralizing antibodies (II). In preliminary studies, treatment with FK506 resulted in dose-dependent allograft survival (Table 6). Treatment with 3.0 mg/kg/d for the first week followed by 1.5 mg/kg/d until 8 weeks (group 7, yellow) resulted in good long-term allograft survival and the development of TxCAD (Figure 19). This model was thus used in the subsequent studies (II).

**Figure 19.** Mouse chronic rejection model. Mouse cardiac allograft recipients were treated with suboptimal FK506 background immunosuppression (group 7 in Table 6) and the allografts were harvested 8 weeks after transplantation. Arteriosclerotic changes were found both in small (A, arrows indicate intima) and larger allograft coronary arteries (B, almost total occlusion). Interstitial and perivascular fibrosis (blue, Masson’s trichrome staining) was also present (C and D). Higher magnification of the boxed area (C) is seen on the lower right panel (D). Haematoxylin-eosin staining in A and B. Masson’s trichrome staining for fibrosis (blue) in C and D.
2. Chronic rejection induces primitive angiogenesis in cardiac allografts (I, II and III)

To characterize angiogenesis in cardiac allografts, antibodies against primitive cells (c-kit, VEGFR-2) and EC (CD31, VEGFR-2) were used. The results are summarized in Figure 20. Only few c-kit+ cells were detected in normal mouse heart. In contrast, numerous c-kit+ cells with capillary morphology were seen in myocardium, adventitia and intima of occluded arteries in chronically-rejecting mouse cardiac allografts (II). The majority of c-kit+ cells co-expressed CD31 and VEGFR-2, and some were positive for the proliferation marker Ki67. A positive correlation was verified between the number of c-kit+ capillaries, and the degree of allograft inflammation and arteriosclerosis (II). Neocapillarization was also detected in the intima of severely occluded rat allograft arteries (IV), and in intimal lesions of rabbit cardiac allograft arteries (I).

3. Cardiac allograft endothelial cells are mainly donor-derived (II)

Marker gene mice and rats were used to determine the origin of cardiac allograft EC and the results are summarized in Figure 21. Endothelial and endomyocardial LacZ expression was detected in non-transplanted Tie1/LacZ rat hearts and the LacZ expression was upregulated in Tie1/LacZ allografts transplanted to wild type (WT) recipients. In contrast, only few LacZ+ capillaries were found in WT cardiac allografts transplanted to Tie1/LacZ recipients indicating that endothelial replacement with recipient-derived cells was rare. Also, the recipient-derived LacZ+ EC localized to severely fibrotic areas in the allografts (II).

To investigate the contribution of recipient BM-derived cells in allograft angiogenesis transgenic mice with constitutive GFP expression in bone marrow cells (GFP-BM) were used as cardiac
Figure 21. Donor origin of cardiac allograft endothelial cells. In Tie1/LacZ allografts transplanted to wild type (WT) recipients diffuse LacZ expression (indicating donor Tie1+ cells) was detected in allograft endothelium (A). In contrast, when WT allografts were transplanted to Tie1/LacZ recipients, only few LacZ+ cells (indicating recipient-derived Tie1+ cells) were detected in severely fibrotic areas (B, arrows). In chronically-rejecting mouse cardiac allografts GFP+ bone marrow-derived cells did not co-express c-kit (C, GFP-BM recipients). In contrast, c-kit+ cells in Balb allografts transplanted to C57 recipients expressed Balb MHC I (D, arrows) indicating donor origin.

allograft recipients. GFP expression was found in allograft CD11b+ myelomonocytic inflammatory cells but not in CD31+ EC or c-kit+ cells indicating that transdifferentiation of recipient BM-derived cells to EC in the allograft was minimal. In addition, donor specific anti-Balb MHC class I antibodies were used to determine the origin of mature and primitive EC in cardiac allografts. Anti-Balb MHC class I was expressed in cardiac allograft CD31+ EC and c-kit+ cells indicating that these cells originate mainly from the donor (Figure 21D). Also, numerous recipient MHC class I+ cells were found around occluded arteries whereas only few positive cells were detected in the intima. In contrast, abundant donor MHC class I immunoreactivity was found in the neointima (II).

4. Expression of vascular growth factors is altered in transplanted hearts (I, II and III)

The expression of VEGF, PDGF, angiopoietins, and their receptors was studied in non-transplanted hearts, syngrafts, and allografts using in situ hybridization and immunohistochemistry. The results are summarized in Table 7.

In normal non-transplanted rat heart, low VEGF mRNA and protein expression was found in cardiomyocytes. During acute rejection, moderate VEGF expression was detected localizing to cardiomyocytes and mononuclear inflammatory cells whereas the expression was mild in syngeneic controls. Moderate VEGF mRNA and protein expression was observed during chronic rejection, and VEGF protein was also seen in media, intima and endothelium of arteries. The degree of TxCAD correlated with VEGF immunoreactivity in allograft inflammatory
cells, cardiomyocytes, endothelium, and media and intima of arteries. In chronically-rejecting cardiac allografts, VEGFR-1 expression was detected in cardiomyocytes, and media and intima of arteries. In contrast, VEGFR-2 was primarily expressed in the endothelium of veins and capillaries.

Only few parenchymal Ang1-immunoreactive cells were found in non-transplanted rat hearts, acute syngrafts and allografts, and chronic syngrafts. No Ang1 immunoreactive cells were found in chronically-rejecting cardiac allografts. Ang2 immunoreactivity was mainly found in the endothelium of capillaries and post-capillary venules, and to lesser extent in cardiomyocytes and allograft-infiltrating mononuclear cells. The expression of Ang2 was increased in chronically-rejecting cardiac allografts. Immunoreactivity for the angiopoietin receptor Tie2 was primarily found in allograft capillaries and postcapillary venules. In severely arteriosclerotic arteries, both Ang2 and Tie2 were expressed in intimal capillaries.

In chronically-rejecting rat cardiac allografts, PDGF-A mRNA and protein were expressed in cardiomyocytes, media of arteries and in some allograft-infiltrating mononuclear cells. PDGF-B mRNA and protein were found in endothelium and inflammatory cells, and PDGF-B protein also in media and intima of arteries. PDGFR-α was expressed in inflammatory cells, and media and intima of arteries. PDGFR-β mRNA and protein were expressed in endothelium, media and intima of arteries.

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EC, endothelial cell; CMC, cardiomyocyte; PCV, post-capillary venule.

### 5. Adenovirus- and AAV-mediated transgene expression in transplanted hearts (I, IV and V)

Intracoronary perfusion with adenovirus or AAV vectors was used to achieve the expression of reporter genes or vascular growth factors in the transplanted hearts. The results are summarized in Figure 22. In rabbit cardiac allografts, adenovirus-mediated myocardial transgene expression was detected in allografts harvested 30 days after the transplantation (I; CsA 3.0 mg/kg/d). In rat cardiac allografts, low to moderate myocardial reporter gene expression was found in allografts 7 days after the transplantation but not 8 weeks after transplantation (IV; CsA 2.0/1.0 mg/kg/d).

In AAV-perfused heart transplants, the control gene luciferase activity increased gradually over time. The transgene expression was diffuse 8 weeks after the transplantation and localized
to cardiomyocytes (V). After intracoronary AAV-perfusion of the heart transplant, transient transgene expression was also detected in the recipient liver at 2 and 3 weeks. In non-transplanted hearts, intramyocardial AAV injection resulted in local transgene expression that remained stable for at least one year (V).

Figure 22. Intracoronary gene transfer in cardiac allografts. Schematic presentation of transgene expression kinetics over time using adenovirus or AAV vectors (A). In rabbit cardiac allografts, LacZ immunoreactivity was detected in the myocardium 30 days after Ad.LacZ perfusion (B). In rat cardiac syngrafts, bioluminescent imaging revealed stable luciferase expression in the transplant (C, arrows) and transient luciferase expression in the liver (C, arrowhead) after intracoronary AAV-Luc perfusion. Diffuse luciferase expression was found in the transplant (D, left) whereas no expression was detected in recipient own heart (D, right). Intramyocardial AAV-Luc injection resulted in localized transgene activity that lasted up to one year (E). In rat cardiac allografts, EGFP immunoreactivity was detected across the whole allograft cross section 8 weeks after AAV-EGFP perfusion (F). EGFP immunoreactivity localized to cardiomyocytes (G, green) along allograft capillaries (G, red).

6. VEGF has pro-inflammatory and pro-arteriosclerotic effects in cardiac allografts (I, II and III)

The functional role of VEGF in the development of TxCAD was studied by adenoviral VEGF gene transfer, or by pharmacological VEGF inhibition with PTK787 or monoclonal antibodies
against VEGFR-1 or -2. The arteriosclerotic and inflammatory (macrophages/myelomonocytic cells) results of different studies are summarized in Figure 23.

Intracoronary perfusion of rabbit cardiac allografts with Ad.VEGF increased the incidence and intensity of arteriosclerosis in medium-sized allograft coronary arteries. Concomitantly, Ad.VEGF perfusion increased the density of allograft-infiltrating macrophages and the incidence of neocapillaries in arteriosclerotic lesions (I). *In vitro*, administration of VEGF resulted in a slight increase in SMC migration but did not induce SMC proliferation (III).

PTK787 was administered daily to study the effect of VEGF inhibition in chronically-rejecting rat cardiac allografts. Treatment with PTK787 decreased TxCAD and macrophage infiltration to the allograft. In addition, PTK787 decreased PDGF-A expression in the media of rat allograft arteries, and ICAM-1 expression in allograft blood vessels (I and III).

The role of the two VEGF receptors was studied using neutralizing anti-VEGFR-1 and -2 antibodies. In the mouse chronic rejection heart transplantation model, treatment with either VEGFR-1 or -2 antibodies profoundly decreased myelomonocyte infiltration and the development of TxCAD. VEGFR-2 inhibition also normalized the density of primitive c-kit+ and mature CD31+ capillaries in allograft myocardium, and was associated with lower allograft mRNA levels of IP-10 and MCP-1 (II).

![Figure 23. The effect of VEGF overexpression and inhibition, and PDGF inhibition on TxCAD and inflammation. The values are in relation to the respective control values (red dashed line). Ref indicates the respective thesis article. Inflammatory cells are RAM11+ (I) or ED1+ macrophages (III) or CD11b+ myelomonocytic cells (II).](image-url)

7. PDGF regulates SMC functions and intimal development in cardiac allografts (III)

The effect of PDGF inhibition on TxCAD was studied using *in vitro* SMC assays and treating rat cardiac allograft recipients with imatinib. *In vitro*, PDGF-B induced strong SMC migration and proliferation that was totally inhibited by imatinib. In this experimental setting without SMC prestimulation, PDGF-A did not induce the migratory or proliferative responses (III). *In vivo*, daily treatment of rat cardiac allograft recipients with imatinib reduced TxCAD
especially together with PTK787 administration. Imatinib alone did not inhibit inflammatory cell infiltration to the allograft, or the immunoreactivity for VEGF, PDGF-A or -B (III).

8. Angiopoietins have common protective and divergent SMC effects in allografts (IV-V)

The functional role of angiopoietins was studied by perfusing rat cardiac allografts with adenovirus vectors encoding Ang1, or AAV vectors encoding Ang1 or Ang2 (Figure 24). In the chronic rejection model, Ad.Ang1 protected against the development of TxCAD and interstitial fibrosis. Ad.Ang1 perfusion reduced the density of macrophages, lymphocytes and immunoactivated IL-2Rα+ cells in the allograft. In addition, Ad.Ang1 perfusion reduced Ang2 expression in microcirculation, and resulted in a transient increase of CD34+ stem cell counts in peripheral blood (IV).

![Figure 24. The effect of adenovirus- or AAV-mediated angiopoietin overexpression on TxCAD and inflammation. The values are in relation to the respective control values. Ref indicates the respective thesis article. Inflammatory cells are ED1+ macrophages.](image)

The effect of prolonged angiopoietin transgene expression in cardiac allografts was investigated by perfusing rat cardiac allografts with AAV vectors (V). AAV-Ang1 and AAV-Ang2 had similar protective but divergent SMC effects. AAV-Ang1 and AAV-Ang2 both reduced the infiltration of macrophages and lymphocytes to the transplanted hearts. This anti-inflammatory effect was associated with a decrease in ICAM-1 and VCAM-1 adhesion protein expression, and an increase in allograft mRNA levels of anti-apoptotic Bcl-2, and Bcl-2/Bax ratio. Only AAV-Ang2 protected against the development of TxCAD whereas AAV-Ang1 perfusion increased allograft SMC activation determined by the expression of pericyte activation marker HMW-MAA, and proliferation marker Ki67 in allograft arteries. In addition, AAV-Ang1 increased the expression of SMC cytokines PDGF-A, PDGF-B and HB-EGF (V).
DISCUSSION

The findings of this study indicate that vascular growth factors have an important role in pathological responses in transplanted hearts. The expression of VEGF (I, II and III), angiopoietins (IV and V) and PDGF (III) was altered in cardiac allografts, and interplay between inflammation, primitive donor-derived capillary angiogenesis, and the development of arteriosclerosis was found (II). Functional studies showed that VEGF has pro-inflammatory, pro-angiogenic and pro-arteriosclerotic effects in cardiac allografts and that these effects can be counteracted by anti-VEGF strategies (I, II and III). Angiopoietins in turn have protective anti-inflammatory, anti-apoptotic and anti-arteriosclerotic effects but prolonged Ang1 exposure may also result in SMC activation (IV and V). Finally, regulating pathological SMC responses with PDGF-inhibition together with EC-targeted interventions may offer better therapeutic tool to prevent inflammation, SMC proliferation and the development of TxCAD (III).

Uncontrolled vascular activation in cardiac allografts

The response-to-injury hypothesis applied to TxCAD suggests that damage to the EC of allograft arteries initiates medial SMC activation and their accumulation to the intima of arteries (Hayry et al. 1993; Ross 1993). This theory has recently been challenged by the heterogenous origin of intimal SMC and the plasticity of stem and progenitor cells during physiological and pathological repair processes (Hillebrands et al. 2001; Saiura et al. 2001; 2003; Quaini et al. 2002; Sata et al. 2002). Previous and present findings (I-V) indicate that in addition to allograft arteries, pathological vascular remodelling also occurs at microvascular level in transplanted hearts (Atkinson et al. 2005; Seipelt et al. 2005).

Therefore, the following modified response-to-injury hypothesis in TxCAD is suggested (Figure 25). According to this hypothesis, the transplanted heart and the allograft recipient respond to different transplantation-related stimuli by activation of alloimmune response together with the activation of cardiac allograft vasculature both at microvascular and arterial level. If the stimulus and activation are controlled and self-terminated, angiogenesis, repair and physiological remodelling may result. In contrast, uncontrolled and prolonged alloimmune and vascular activation in a transplanted heart leads to interplay of inflammation, angiogenesis, vascular wall dysfunction and intimal growth. Stem and progenitor cells participate in the physiological and pathological reparative responses, and may originate either from the donor or the recipient depending on the degree of injury present.

Pro-inflammatory, pro-angiogenic and pro-arteriosclerotic effects of VEGF in cardiac allografts

VEGF regulates essential developmental functions and is a therapeutic target in situations with both excess angiogenesis, and inadequate vascularization, and in several inflammatory conditions (Ferrara et al. 2003; Tammela et al. 2005). VEGF expression is increased during acute and chronic rejection in transplanted hearts, and VEGF is mainly produced by allograft inflammatory and parenchymal cells (Torry et al. 1995; Reinders et al. 2003a) (I, II and III). As serum VEGF levels are increased during acute rejection (Abramson et al. 2002) VEGF may elicit functional responses both locally in the transplanted heart, and also in distant recipient tissues.
Figure 25. Uncontrolled activation of repair processes and pathological responses leading to TxCAD. Normal cardiac vasculature is in a quiescent state. During and after heart transplantation, controlled activation and downregulation of angiogenesis, repair and remodelling may result in physiological responses. In contrast, several stimuli related to the heart transplantation may trigger an uncontrolled repair process that involves interplay between vascular wall dysfunction, inflammation, angiogenesis and intimal growth. Both mature EC and SMC, and vascular stem and progenitor cells participate in the physiological and pathological repair mechanisms. The origin of these cells is probably heterogeneous and reflects the degree of allograft injury. Acute Rx, acute rejection; CMV, cytomegalovirus infection; EC, endothelial cell; I/R, ischaemia-reperfusion injury; SMC, smooth muscle cell; TxCAD, transplant coronary artery disease.

VEGF expression is regulated both by hypoxic (Shweiki et al. 1992) and inflammatory pathways (McCourt et al. 1999). This suggests that both ischemic and alloimmune stimulus in cardiac allografts participate in VEGF production. The proposed functional role of VEGF in cardiac allografts is presented in Figure 26 on the basis of current and previous transplantation studies together with the described VEGF effects in non-transplant situations. Although VEGF has protective and reparative effects in vascular injury (Asahara et al. 1995), it has several potent pro-inflammatory and pro-arteriosclerotic effects in cardiovascular disease (Celletti et al. 2001b; Bhardwaj et al. 2005a), and after heart transplantation.

Outside the cardiac allograft, VEGF may have important effects on alloimmune activation and inflammatory and vascular progenitor cell trafficking. VEGF regulates the BM capillary milieu and facilitates mobilization of inflammatory cells and vascular progenitor cells to the peripheral circulation (Hattori et al. 2001). In lymph nodes, VEGF-producing dendritic cells increase angiogenesis (Webster et al. 2006). Dendritic cells can also transfer NRP1 and VEGF.
to T cells during antigen presentation (Tordjman et al. 2002; Bourbie-Vaudaine et al. 2006). Therefore, VEGF has important effects during antigen presentation and lymphocyte trafficking and thus during alloimmune reactions, although it does not directly induce T cell proliferation (Reinders et al. 2003b). Several studies also indicate that VEGF is a direct chemotactic signal for myelomonocytic cells through VEGFR-1 (Clauss et al. 1990; 1996; Barleon et al. 1996; Luttun et al. 2002). As VEGFR-1 inhibition profoundly reduced myelomonocyte cells in the allograft – while VEGFR-1 expression in allograft inflammatory cells was low (II) – VEGFR-1 seems to regulate leukocyte recruitment to allografts principally at BM and peripheral blood level.

In the allograft, VEGF increases inflammation primarily through allograft EC-mediated effects. In the IFN-γ-containing pro-inflammatory allograft milieu, VEGF induces EC to produce chemokines IP-10 and MCP-1 (Boulday et al. 2006). This, in turn, facilitates the recruitment of T cells and monocytes, respectively (Reinders et al. 2003b). VEGF also induces the expression of endothelial adhesion proteins (Kim et al. 2001b), and increases vascular permeability (Weis et al. 2004). As this study demonstrated that VEGFR-2 – but not VEGFR-1 – was primarily expressed in the endothelium of allograft capillaries and postcapillary venules (II and III), VEGFR-2 is probably largely responsible for the EC-mediated pro-inflammatory effects of VEGF in cardiac allografts.

Interestingly, the current observations also indicate that VEGFR-2 is important in capillary angiogenesis in the allograft (II). The VEGFR-2+ capillaries expressed the stem cell marker c-kit and the allograft EC were almost exclusively donor-derived. These findings suggest that the primitive allograft capillaries originated either from resident stem or primitive cells such as the recently-described CSC (Anversa et al. 2006), or alternatively, chronic rejection may have induced mature allograft EC to adopt a more primitive phenotype and the expression of c-kit and VEGFR-2. In any case, the current results imply that VEGF is involved in the interplay of inflammation and primitive angiogenesis (II). As VEGFR-2 inhibition reduced capillary angiogenesis and inflammation (II), cardiac allograft angiogenesis may directly relate to increased inflammatory cell recruitment. Supporting this view, immature new blood vessels are usually permeable until proper connections with underlying supporting cells are established (Thurston et al. 1999; Hellstrom et al. 2001). Moreover, as angiogenesis in cardiac allografts did not associate with EC replacement with recipient-derived cells (II) (Hillebrands et al. 2001) the formation of new primitive blood vessels may not offer any immunological advantages.

VEGF also mediates pro-arteriosclerotic processes in cardiac allografts (I, II and III). The effect of VEGF on intimal lesion progression may involve perivascular inflammation, adventitial and intimal angiogenesis, and direct or endothelial-mediated effects on mature and immature SMC. Recent reports suggest that angiogenesis in the adventitia and intima of cardiac allograft arteries is linked to the progression of intimal growth (Atkinson et al. 2005; Seipelt et al. 2005). The current studies also demonstrate the presence of immature capillaries in the adventitia and intima of cardiac allograft arteries (II). Moreover, adventitial and intimal angiogenesis may play a regulatory role in intimal growth as VEGF overexpression induced intimal capillarization and TxCAD (I), and VEGF-inhibition reduced TxCAD (I-III). In a non-transplant rabbit model, application of VEGF to arterial adventitia resulted in intimal growth, whereas application
Figure 26. The proposed role of VEGF in TxCAD. VEGF increases angiogenesis in BM and secondary lymphoid organs, and mobilizes inflammatory cells and vascular progenitor cells to peripheral circulation. Inflammatory cells and allograft cardiomyocytes are the main sources of VEGF in cardiac allografts. VEGF directly enhances the recruitment of VEGFR-1+ myeloid cells to the allograft, and increases leukocyte extravasation at microvascular level especially in the pro-inflammatory cardiac allograft microenvironment. VEGFR-2 is possibly the principal VEGF-receptor mediating the production of endothelial adhesion proteins and chemokines, and increased vascular permeability. In response to transplantation, VEGF initiates reparative processes in the allograft by inducing angiogenesis that may originate from resident mature EC, vascular progenitor or stem cells derived from resident or circulating cells. The newly found vessels may be permeable and thus increase the inflammatory response. VEGF-induced adventitial and intimal angiogenesis may be a driving force for intimal growth in allograft arteries. VEGF may also contribute to TxCAD by activating arterial and intimal SMC through endothelium-derived cytokines, or directly by inducing VEGFR-1+ SMC migration. APC, antigen-presenting cell; BM, bone marrow; CSC, cardiac stem cell; EC, endothelial cell; EPC, endothelial progenitor cell; HSC, haematopoietic stem cell; LN, lymph node; MMP, matrix metalloproteinase; NRP, neuropilin receptor; SMC, smooth muscle cell.
of Ang increased neocapillarization but did not result in intimal development (Bhardwaj et al. 2005a; 2005b). Therefore, functional differences in neocapillary function and not only the growth of new capillaries may have an impact on the following physiological and pathological responses in arteries.

In addition to adventitial and intimal angiogenesis, VEGF may induce pro-arteriosclerotic effects by direct or indirect effects on SMC and their progenitors. VEGF induces the expression of SMC cytokines from EC (Kano et al. 2005) and the current results show that allograft PDGF production was decreased during VEGF-inhibition (II and III). VEGF also mobilizes and recruits vascular progenitor cells that may subsequently transdifferentiate to SMC (Hattori et al. 2001; Simper et al. 2002) and possibly participate in intimal development. VEGF is also a direct chemoattractant for VEGFR-1+ SMC (Couper et al. 1997; Wang and Keiser 1998; Grosskreutz et al. 1999; Shibata et al. 2001; Parenti et al. 2002; Parenti et al. 2004) and may thereby recruit VEGFR-1+ SMC (II and III) from the media to the developing intima.

**Anti-inflammatory and anti-arteriosclerotic effects of angiopoietins in cardiac allografts**

Angiopoietins have important direct endothelial and indirect SMC effects in vascular maturation and maintenance (Eklund and Olsen 2006). The present study showed that Ang1 expression was low in transplanted heart and was further decreased in chronically-rejecting cardiac allografts (IV). In contrast, endogenous Ang2 immunoreactivity was increased in microvascular endothelium (IV) supporting the recent finding that Ang2 is an autocrine protective factor in stressed EC (Daly et al. 2006). Also the angiopoietin receptor Tie2 was detected in allograft capillaries (IV). As no prior information exists on the role of angiopoietins in transplanted organs, the proposed effects of Ang1 and Ang2 in heart transplants are illustrated in Figure 27 based on the present findings (IV and V) and previous non-transplantation studies. Angiopoietins may have important anti-inflammatory, anti-apoptotic, anti-arteriosclerotic and protective effects in cardiac allografts while Ang1 may also increase SMC activation.

Outside the allografts, Ang1 is involved in the mobilization of inflammatory cells, and haematopoietic and vascular progenitor stem cells from BM to peripheral circulation (Hattori et al. 2001). This study also documented a transient increase in peripheral CD34+ cell counts after adenoviral Ang1 gene transfer (IV). In addition to Ang1, also Ang2 is involved in the differentiation and migration of circulating EPC (Hildbrand et al. 2004; Gill and Brindle 2005).

In the allograft, both Ang1 and Ang2 had anti-inflammatory and anti-apoptotic effects (IV and V). The protective effects of Ang1 may include anti-apoptotic effects (Kwak et al. 1999; Kim et al. 2001a; Chen et al. 2004a) through increased PI3K /Akt signalling and decreased ABIN-2-mediated NF-kappa-β signalling (Kim et al. 2000a; Hughes et al. 2003; Tadros et al. 2003; DeBusk et al. 2004). In addition, Ang1 may reduce vascular permeability (Thurston et al. 1999; 2000; Visconti et al. 2002; Arsic et al. 2003; Baffert et al. 2006), EC adhesion protein expression (Kim et al. 2001c) and tissue factor expression (Kim et al. 2002). Ang1 may also increase cardiomyocyte survival through Tie2-independent and integrin-mediated mechanisms (Dallabrida et al. 2005) possibly also in cardiac allografts.
Figure 27. The proposed role of angiopoietins in TxCAD. Angiopoietins mobilize inflammatory and progenitor cells to peripheral circulation. At capillary level, exogenous Ang1 and Ang2 have vasculoprotective and anti-inflammatory effects. Ang1 also increases the production of SMC cytokines from EC. In addition to Tie2- and endothelial-mediated effects, angiopoietins have integrin-mediated anti-apoptotic effects in cardiomyocytes. At the arterial level, both Ang1 and Ang2 inhibit intimal thickening probably through beneficial effects on inflammation and adventitial and intimal angiogenesis. Ang1 also activates medial and intimal SMC and thereby may actually contribute to intimal thickening. BM, bone marrow; CSC, cardiac stem cell; EC, endothelial cell; HSC, haematopoietic stem cell; LN, lymph node; SMC, smooth muscle cell.
As Ang2 had protective effects in the present study (V), this ligand may activate Tie2 signalling in cardiac allografts. This is interesting as it was first thought that Ang2 is an endogenous competing Ang1 antagonist (Maisonpierre et al. 1997). In contrast, several subsequent findings indicate that Ang2 – at least in some circumstances – activates Tie2 signalling and elicits potential anti-apoptotic (Kim et al. 2000b; Chen et al. 2004a) and anti-permeability effects (Daly et al. 2006). Similarly to Ang1, also Ang2 binds to integrins (Carlson et al. 2001) and mediates myocyte adhesion Tie2-independently (Dallabrida et al. 2005).

The anti-arteriosclerotic effects of angiopoietins in cardiac allografts probably result in part from the anti-inflammatory properties of the ligands. Ang1 stimulation may result in stronger physiological adventitial capillarization that inhibits intimal formation (Bhardwaj et al. 2005b). Ang2 may also inhibit intimal neovascularization (Calvi et al. 2004). The current results show that in contrast to transient adenoviral-mediated Ang1 expression, prolonged AAV-mediated exposure to Ang1 increased SMC cytokine production, activated allograft SMC and did not have a beneficial effect on TxCAD. Therefore, the SMC activation induced by prolonged Ang1 exposure may actually counteract the beneficial anti-inflammatory effects of Ang1. Similarly, Ang1 protects pulmonary endothelium and inhibits the development of pulmonary hypertension (Zhao et al. 2003) but increased SMC cytokine production may actually result in the progression of the disease (Sullivan et al. 2003; Chu et al. 2004). Thus, the timing and the environment of Ang1 exposure are probably important in the balance of beneficial and harmful effects in cardiac allografts. In contrast to Ang1, AAV-Ang2 did not result in SMC activation in cardiac allografts which is consistent with previous reports showing that Ang2 does not induce SMC cytokine production from EC (Iivanainen et al. 2003). Therefore, Ang2 may have a better therapeutic profile than Ang1 in cardiac allografts at least with prolonged administration.

Anti-arteriosclerotic and SMC-activating effects of PDGF in cardiac allografts

PDGF-ligands are potent factors mainly for mesenchymal cells. Figure 28 summarizes the effects of PDGF in cardiac allografts based on previous, current (III) and unpublished results together with non-transplantation studies. PDGF ligands probably elicit pro-arteriosclerotic effects through migratory and proliferative effects on SMC (Sihvola et al. 1999). In addition, PDGF may recruit monocytes (Uutela et al. 2004), and circulating and resident vascular progenitor cells and guide their differentiation to SMC cells (Yamashita et al. 2000; Simper et al. 2002). Our previous and current findings indicate that PDGF-B results in strong SMC proliferation and migration in vitro (III), whereas PDGF-A has effects on SMC only after inflammatory cytokine stimulation (Sihvola et al. 1999). Previous findings also show that PDGF-A/PDGFR-α signalling is an important regulator of fibroproliferative responses in transplanted hearts (Mancini and Evans 2000). Our unpublished results on the effects of different PDGF-ligands (PDGF-A-D) indicate PDGF-A/C/PDGFR-α signalling as the main mediator for inflammation, fibrosis and intimal growth in cardiac allograft while PDGF-B/PDGFR-β signaling resulted only in physiological vascular effects. These findings highlight the pathological role of PDGFR-α in the inflammatory heart transplant environment whereas PDGFR-β signalling may predominate in arterial injury models and atherosclerosis (Raines 2004).
Figure 28. The proposed role of PDGF in TxCAD. Endothelial cells, platelets and inflammatory cells are the major source for PDGF in cardiac allografts. PDGF may recruit PDGFR+ monocytes and vascular progenitor cells from the bone marrow to the allograft. PDGF primarily regulates the migration and proliferation of SMC to the intima of allograft arteries, and is a mitogen for cardiac allograft fibroblasts. PDGF may also guide the recruitment and differentiation of circulating or resident vascular progenitors to SMC. BM, bone marrow; CSC, cardiac stem cell; EC, endothelial cell; HSC, haematopoietic stem cell; LN, lymph node; SMC, smooth muscle cell.
Implications for translational studies

Uncontrolled activation of vascular cells in cardiac allografts results in pathological processes including interplay of inflammation, angiogenesis, intimal growth, and repair processes involving stem and progenitor cells. The current medical therapy in heart transplant patients targets mainly at clonal proliferation of alloreactive T cells (Lindenfeld et al. 2004a). Although new immunosuppressive regimens effectively prevent acute rejections, the development of TxCAD and side-effects of the immunosuppressive drugs remain a significant problem. Therefore, strategies that target at other pathways than directly the T cell proliferation are warranted.

This study and previous findings indicate altered vascular biology in transplanted organs as a potent target for therapeutic intervention. Specifically, the current results imply that either VEGF- or PDGF-inhibition, or angiopoietin therapy may result in anti-inflammatory and anti-arteriosclerotic effects in cardiac allografts and that these pathways may be targeted either by systemic drugs or local gene therapy. In favour of vascular structures as therapeutic targets in cardiac allografts, immunosuppressants MMF, sirolimus and everolimus that also control SMC proliferation have shown beneficial effects on the development of TxCAD (Eisen et al. 2003; Mancini et al. 2003; Kobashigawa et al. 2006).

Recent advances in vascular and cancer biology have produced several pharmacological agents that target at EC and SMC and could also be used in the setting of solid organ transplantation (Jain et al. 2006), and some of these drugs such as imatinib (Buchdunger et al. 2002), and the anti-VEGF antibody bevacizumab (Hurwitz et al. 2004) are already in clinical use. Before clinical studies in transplant patients can be initiated, several things should be considered. First, the results of small animal models should be validated in larger animal models. Second, specific therapeutic windows for pharmacological intervention should be assessed as the drugs used may have unwanted side-effects especially if continuous therapy is applied (Kerkela et al. 2006). Third, the pathological processes after heart transplantation should be better known at molecular level to achieve more targeted therapies. Fourth, contrasting the previous notion, preventing and treating pathological processes in cardiac allografts may require simultaneous targeting of several key pathways as demonstrated with the current results using combined VEGF and PDGF inhibition.

In addition to pharmacological approaches, gene therapy could be an ideal therapeutic option in organ transplantation. Gene vectors could be easily applied during the ex vivo time of the transplanted organ and systemic side-effects could be avoided with local production of the therapeutic transgene. Several vector- and transgene-related factors should be considered before gene therapy is applied in the clinical setting. Different gene vectors have different properties of transduction efficiency, tissue and cell tropism, transgene expression kinetics, evoking of host inflammatory responses, ease of production and transgene packaging ability (Thomas et al. 2003). Therefore, the same therapeutic gene administered with one vector could have completely different results if administered with another vector. As an example of this, the current results indicate different results on SMC activation with adenoviral- or AAV-mediated Ang1 gene transfer (IV and V). These observations may at least in part be explained by the expression kinetics of these vectors as adenovirus results in rapid but transient expression (Fujishiro et al. 2005) whereas the current results show that AAV vectors result in stable transgene expression in transplanted hearts (V). Also, the therapeutic transgene should
be carefully chosen while considering the vector properties and the pathobiology of the disease involved. Currently, gene vectors offer a powerful tool to investigate pathological processes and therapeutic molecules in experimental transplantation models. The recent progress in gene vector biology (Pacak et al. 2006) together with improved knowledge of pathological processes in transplanted organs suggest that gene therapy may be a safe and efficient way to introduce therapeutic genes also in clinical transplantation in the future.

Conclusions

The findings of these studies indicate an interplay of inflammation, angiogenesis and arteriosclerosis in cardiac allografts, and show that vascular growth factors are important regulators in the process. Particularly, VEGF inhibition, PDGF inhibition and angiopoietin therapy with clinically relevant pharmacological agents or novel gene therapy approaches may counteract vascular dysfunction in cardiac allografts, and have beneficial effects on the survival of heart transplant patients in the future.
YHTEENVETO (FINNISH SUMMARY)

Sydänsiirto on usein ainoa mahdollinen hoitomuoto loppuvaiheen sydäntautia sairastavalle potilaalle. Vaikka lyhyaikaikaisennuste on parantunut huomattavasti, sydänsirtoon liittyvät immunologiset ja ei-immunologiset tekijät lisäävät sydänsiirteen sepelvaltimotaudin kehittymistä ja tällä hetkellä terapeuttiset keinot ovat vähäiset.

Nykytiedon valossa sydänsiirteen sepelvaltimotaudin johtuu siirteen verisuonun huonosti säädellystä korjausreaktiosta, johon oleellisesti kuuluu verisuonen seinänmuutosta epänormaali toiminta. Aktioidut sydänsiirteen endoteelisolot toimivat antiinflammatorisesti verisuonin vahingoittamiseen virtaen ja erittävät silälihassolujen kasvua edistävää kasvutekijöitä. Silälihassolot ja niiden kantamuotot puolestaan keräävät vaurioituneen sepelvaltimon intimaan, mikä lopulta johtaa arterian tukkeutumiseen.


Tutkimuksessa osoitettiin, että sydänsiirteen hyljintäreaktiio lisää VEGF tuotantoa pääasiassa siirteen makrofageissa ja aiheuttaa verisuonun uudismuodostusta siirteessä. Siirteen angiogeneesiin osallistui primitiivisitä endoteelisoluja, jotka olivat päätös peräisin sydänsiirteen soluista. VEGF geenisiirto adenovirusvektorilla lisäsi makrofagien määrää, intiman uudissuonimuodostusta ja siirteen arterioskleroaosia. VEGF esto tyroksiininikasain estäjä PTK787:lla vähensi siirteen tulehdusta ja arterioskleroaosia, ja samanaikainen PDGF esto imatinibilla edelleen vähensi arterioskleroaosia. Selektiivinen kahden eri VEGF-reseptorin (VEGFR-1 ja -2) esto monoklonaalisilla vasta-aineilla vähensi selkeästi siirteen tulehdusta ja sepelvaltimotautuutta, ja VEGF-2 esto lisäksi normalisoi kypsien ja epäkypsien kapillaarisoheen määrän. Nämä tulokset viittaavat siihen, että VEGF esto voitaisiin käyttää uudenlaisena hoitoon siirteen tulehdusta ja arterioskleroaosia myös kliinisessä tilanteessa, ja samanaikaisella PDGF estolla voisi olla suotuisa vaikutus silälihassolujen normalisointiin kasvuvan.

Toisin kuin VEGF-geenisiirrolla, adenovirusvälisteisellä lyhytaikaisella Ang1 geenituotannolla oli siirteen tulehdusta ja arterioskleroaosia estävä vaikutus. Adeno-associated virus (AAV)-välisteisellä pitkäaikaisella Ang1 tai Ang2 geenituotannolla oli molemmilla samannäin anti-inflammatorinen vaikutus. Pitkäaikaiset Ang1 geenituotanto kuitenkin lisäsi siirteen silälihassolujen aktivointiin, kun taas Ang2 geenituotanto ei aktivointin silälihassolujen ja vähensi sydänsiirteen sepelvaltimotautia. Tulokset antavat viitteitä siitä, että angiopoietiineillä voisi olla tärkeitä tulehdusta estäviä ja suojavaiavia vaikutuksia myös
sydänsiirtopotilailla, mutta pitkittynyt altistuminen Angl kasvutekijälle saattaa lisätä siirteen patologista sileälhassoluaktivaaatiota. Lisäksi, AAV vektoreita voitaisiin käyttää saamaan aikaan pitkääikainen terapeuttisen geenin ilmentyminen sydänsiirteissä.

Tutkimuksen tulokset tukevat tulehduksen, verisuonten uudismuodostuksen ja sepelvaltimo- taudin yhteyttä sydänsiirteissä ja verisuonten kasvutekijöiden roolia näissä tapahtumissa. Lisäksi tutkimuksen perusteella VEGF esto, PDGF esto ja angiopoietiiniterapia käyttämällä klinisesti relevanteja lääkeaaineita tai geeniterapiaa voi vähentää sydänsiirteen verisuonten solujen epänormaalta toimintaa ja parantaa sydänsiirtopotilaiden pitkääikaisselviytymistä tulevaisuudessa.
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