MICROVASCULAR DYSFUNCTION IN ISCHEMIA-REPERFUSION IN CARDIAC AND KIDNEY ALLOGRAFTS

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Academic Dissertation
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To Erika, Aurora and Adrian
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ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which will be referred to in the text by their Roman numerals:

I

II

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ABBREVIATIONS

3HR three-drug hormonal resuscitation
AAV adeno-associated virus
Ad adeno virus
Ang angiopoietin
α-SMA alpha-smooth muscle actin
BMP bone morphogenetic protein
CAV cardiac allograft vasculopathy
CD cluster of differentiation
CMC cardiomyocyte
CPP cerebral perfusion pressure
CsA cyclosporine A
DA Dark Agouti rat
DC dendritic cell
DGF delayed graft function
EC endothelial cell
ECM extracellular matrix
ECMO extracorporeal membrane oxygenation
EMT epithelial-mesenchymal transition
EndMT endothelial-mesenchymal transition
ET-1 endothelin-1
FITC fluorescein isothiocyanate
FSP fibroblast specific protein
GBM glomerular basement membrane
GGPP geranylgeranyl pyrophosphate
HA hyaluronan
HAS 1-3 hyaluronan synthases 1-3
HIF hypoxia-inducible factor
HMG-CoAR 3-hydroxy-3-methylglutharyl-coenzyme A reductase
HMVEC-C human cardiac microvascular EC
HO heme oxygenase
IABP intra-aortic balloon pump
ICH intracerebral hemorrhage
ICP intracranial pressure
iNOS inducible NO synthase
IRI ischemia-reperfusion injury
kDA kilodalton
L-NAME N-nitro-L-arginine methyl ester
LPS lipopolysaccharide
MAP mean arterial pressure
MHC major histocompatibility complex
MMP matrix metalloproteinase
MP methylprednisolone
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-B</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>p-adducin</td>
<td>adducin phosphorylated at Thr445</td>
</tr>
<tr>
<td>Pd</td>
<td>podocyte</td>
</tr>
<tr>
<td>PDGF A-D</td>
<td>platelet derived growth factor ligands A, B, C and D</td>
</tr>
<tr>
<td>PDGFR α-β</td>
<td>platelet derived growth factor receptors α and β</td>
</tr>
<tr>
<td>p-ERM</td>
<td>phosphorylated form of ezrin/radixin/moesin proteins</td>
</tr>
<tr>
<td>PGD</td>
<td>primary graft dysfunction</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>p-Smad2</td>
<td>phosphorylated Smad2</td>
</tr>
<tr>
<td>Rac</td>
<td>a subfamily of the Rho family of GTPases</td>
</tr>
<tr>
<td>RECA-1</td>
<td>rat EC antigen-1</td>
</tr>
<tr>
<td>RhoA</td>
<td>a member of Rho family GTPases</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>SAH</td>
<td>subarachnoid hemorrhage</td>
</tr>
<tr>
<td>T2</td>
<td>transverse relaxation time</td>
</tr>
<tr>
<td>TAH</td>
<td>total artificial heart</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TnT</td>
<td>cardiac troponin T</td>
</tr>
<tr>
<td>Tx</td>
<td>transplantation</td>
</tr>
<tr>
<td>VAD</td>
<td>ventricular assist device</td>
</tr>
<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>vascular endothelial cadherin</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>vSMC</td>
<td>vascular smooth muscle cell</td>
</tr>
<tr>
<td>WF</td>
<td>Wistar Furth rat</td>
</tr>
<tr>
<td>ZnPP</td>
<td>zinc protoporphyrin</td>
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ABSTRACT

Transplant ischemia/reperfusion injury (Tx-IRI) remains among the major clinical challenges in organ transplantation. Tx-IRI may result in deleterious short-term consequences such as primary graft dysfunction and increased immunogenicity of the allograft, both of which enhance the probability for late vascular remodeling and fibroproliferative processes, ultimately leading to untreatable chronic allograft dysfunction and compromised long-term survival. The underlying mechanisms in primary graft dysfunction involve microvascular dysfunction culminating in increased vascular permeability, perfusion defects, and leukocyte infiltration into the allograft, which may lead to pro-inflammatory and pro-fibroproliferative processes.

The pleiotropic, cholesterol-independent vasculoprotective effects of statins, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, have been well described. Statins improve microvascular integrity and perfusion through endothelial cell (EC) and pericyte function. Statins attenuate the expression and secretion of angiogenic growth factors and microvascular reactivity at the site of vascular injury. Statins seem to have also anti-inflammatory, -oxidative, and -thrombotic effects. They are widely used for primary and secondary prevention of cardiovascular disease. In the transplant recipients, statin treatment decreases allograft inflammation and vasculopathy and cardiovascular morbidity. However, the therapeutic potential of donor statin treatment against Tx-IRI and microvascular dysfunction remains undelineated.

The majority of potential cardiac allograft donors from brain-dead donors do not have previous medical track record of cardiovascular diseases nor statin medication. The heart is especially susceptible to donor brain death that elicits cardiotoxic pro-inflammatory cytokine release, cardiovascular disintegration and poor organ perfusion, which often lead to disqualification of a transplant. On the other hand, shortage of donors and different donor-related factors limit the availability of transplants, and thus lead to the use of organ donors with extended criteria such as older age. Aggressive donor management could not only improve the quality of donated organs, but also expand the donor pool by increasing suitability of donors with extended criteria and thus reduce costs of transplantation affecting e.g. need for inotropic support and stay at intensive care unit. Based on these clinically relevant issues, we chose pharmacological approaches to treat donors to improve allograft resistance to Tx-IRI and primary and chronic allograft dysfunction.

Donor rats without brain death were treated with a single peroral dose of lipophilic simvastatin two hours before heart and kidney removal, which is the clinical time-window to treat a brain-dead organ donor. The cardiac allografts were subjected to 4-h and kidney allografts to 16-h cold-ischemic preservation to mimic clinical situation and transplanted to fully major histocompatibility complex (MHC)-mismatched WF rat recipients. As our current clinical practice includes donor treatment with high dose of methylprednisolone, that modulates inflammatory state after brain death, we also investigated whether combined donor simvastatin and methylprednisolone treatment could be superior to either treatment alone on Tx-IRI and allograft survival.

Here, we report that the expression of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a target molecule of statins, was abundant in endothelial cells (ECs) and pericytes of normal hearts as well as in glomerular and peritubular microvascular structures of normal
kidneys. Rat cardiac and kidney allograft Tx-IRI resulted in profound microvascular
dysfunction; leakage, perfusion defects and increased adhesivity.

Donor, but not recipient treatment with peroral single-dose simvastatin two hours before graft
procurement inhibited microvascular EC and pericyte RhoA/Rho-associated protein kinase
activation and inter-EC gap formation, vascular leakage, the no-reflow phenomenon, danger-
associated ligand hyaluronan induction, leukocyte infiltration and myocardial and
tubulointersitital injury. In the chronic rejection model, donor simvastatin treatment inhibited
cardiac allograft inflammation, TGF-β1 signaling and myocardial fibrosis, vasculopathy and
improved long-term allograft survival. Furthermore, donor treatment with a combination of
simvastatin and methylprednisolone was superior in the prevention of Tx-IRI and
significantly prolonged acute survival of non-immunosuppressed major MHC-mismatched
cardiac allograft.

In conclusion, donor treatment may target microvascular dysfunction, immunomodulation
and the initiation of pro-inflammatory and pro-fibroproliferative pathways in cardiac and
kidney allografts subjected to prolonged ischemia time using a protocol relevant for clinical
cadaveric transplantation. Minimizing microvascular injury and the activation of innate
immunity by combined donor simvastatin and methylprednisolone treatment may offer a
novel therapeutic strategy to expand the donor pool and furthermore improve the function of
donor organs with extended criteria. We have therefore initiated a randomized clinical trial to
investigate the effect of combined donor simvastatin and methylprednisolone treatment as an
adjunct therapy on short- and long-term results of cardiac and kidney allografts.
INTRODUCTION

Allograft organ transplantation means removing an organ from one body (donor) and implanting it to a recipient of the same species for the purpose of replacing the recipient's diseased or damaged organ. Allografts can be either from living, brain-dead or cardiac-dead donor origin. Transplanted solid organs (listed here from the most frequently transplanted to the rarest) are kidneys, liver, heart, lungs, pancreas and intestine.

Shortage of organs limits clinical heart and kidney transplantation. Sixty to 80 patients per million inhabitants per year would benefit from cardiac transplantation (Kottke et al. 1990; Costanzo et al. 1995), but only a quarter of them are placed on the waiting list and one-tenth will receive a cardiac transplant (Rosengard et al. 2002). Up to two thirds of cadaver donor hearts are being discarded (Zaroff et al. 2002; Johnson et al. 2010). Also, the wait-listed number of kidney transplant candidates continues to increase annually. Despite high rates of kidneys recovered for transplant, increasing incidences of those organs are eventually been discarded due to failure to meet transplant criteria (Matas et al. 2012). Optimistic estimations highlight that up to 92% of organs that fail to meet cardiac transplantation criteria on initial evaluation could be functionally resuscitated with aggressive pharmacological donor management (Wheeldon et al. 1995; Rosendale et al. 2003a). Improving the quality of donor organs is also an important economical issue when considering faster weaning from inotropic support and shortened need for critical care bed stay (Marasco et al. 2007).

Donor management is indeed a critical step for organ transplantation. In spite of preservation techniques exploited at procurement and for transportation, brain-death predisposes cardiac and kidney allografts to ischemia-reperfusion -induced rapid alloantigen-independent injuries. These innate immune injuries involve a cytokine storm and hemodynamic instability during brain death, metabolic changes at loss of oxygen and nutrient supply and finally reoxygenation that increases the myocardial injury induced by ischemia alone (Hearse 1977). By the danger model -theory, the alarm signals or damage-associated molecular patterns released by damaged cells are the fundamental process for the activation of antigen-presenting cell (APC) that ultimately controls the balance between host-versus-graft disease and tolerance (Matzinger 1994; Anderson et al. 2001; Matzinger 2002). Prolonging cold-ischemic preservation augments vascular injury independently from allograft immunogenicity. Overall, ischemia time of the donor allograft is linked to the early development of delayed graft function (DGF) in kidney allografts and primary graft dysfunction (PGD) in cardiac allografts. Further, ischemia time induces the progression of chronic allograft dysfunction and decreases late survival of the allografts in a linear fashion.

Pharmacological strategies of the immunosuppressive drugs have been primarily designed to target T and B cell -mediated adaptive immune responses (Bierer et al. 1993; Chen et al. 1993; Fulton and Markham 1996; Berard et al. 1999). The major problems concerning long-term immunosuppressive drugs are their many metabolic, cardiovascular, infectious, and malignant side effects. Moreover, T and B cell-targeted anti-inflammatory therapies generally fail to inhibit the development of allograft fibrosis and vasculopathy. Thus, donor targeted drug therapies aiming to prevent danger-associated molecule expression and microvascular dysfunction hold promise as a new approach for the prevention of cardiac and kidney allograft rejection. Moreover, donor treatment exclusively targeting the transplant eliminates systemic side effects and has no risks of low drug compliance. Statins, HMG-CoA reductase inhibitors, have cholesterol-independent microvasculoprotective effects through cytoskeletal rearrangement of endothelial cells and their surrounding pericytes.
In this study, we used simvastatin to approach donor management in experimental heart and kidney transplantations. Donor simvastatin treatment could offer novel clinically feasible immunomodulatory and microvasculature-targeted adjunct therapy for counteraction of IRI without inflicting harmful side effects induced by conventional immunosuppressive medication.
<table>
<thead>
<tr>
<th>Transplantation terminology</th>
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<tr>
<td>Term</td>
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<tr>
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<tr>
<td>Acute rejection</td>
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<tr>
<td>Chronic rejection</td>
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<tr>
<td>Rejection</td>
</tr>
<tr>
<td>Graft-versus-host disease (GVHD)</td>
</tr>
<tr>
<td>Immunosuppression</td>
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<tr>
<td>T-cell depletion</td>
</tr>
<tr>
<td>Antibody-mediated rejection</td>
</tr>
<tr>
<td>Antibodies</td>
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<tr>
<td>Immune</td>
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REVIEW OF THE LITERATURE

1. Clinical heart and kidney transplantation

Introduction

Already in the 16th century, Italian surgeon Gasparo Tagliacozzi performed successful skin autografts (a graft taken from the same individual) but consistently failed with allografts (a graft taken from an individual of the same species, but of different genotype), and finally came to the conclusion about the "force and power of individuality" in his work *De curtorum chirurgia per insitionem* (1597).

![Figure 1. De curtorum chirurgia per insitionem, 1597](image)

Alexis Carrel, after his skillful operations with arteries or veins, performed the first successful experimental kidney transplantations in the early 20th century and laid the basis for later transplant surgery (Carrel and Guthrie 1905, 1906). Improving donor preservation, surgical techniques and introduction of immunosuppression were needed to achieve acceptable long-term survival (Shumacker 1994).

The first successful transplant in man was performed by Joseph Murray in Boston at Peter Bent Brigham Hospital (currently Brigham and Women’s Hospital) in 1954 (Guild et al. 1955). This renal transplantation took five and half hours and was performed between genetically identical twins to eliminate any problems of immune reaction. Cortisone medication that was used to prevent and treat acute rejections enabled organ transplantations from cadaveric sources. Kidney was the most approachable organ for transplantation because it was relatively easy to remove and implant, tissue typing was easily manageable and in the case of allograft failure, dialysis was available. Success with the kidney led to attempts with other organs.

The exhaustive development of the heart-lung machine, solving perfusion issues and developing surgical techniques by Shumway and Lower at Stanford University enabled successful heart transplantations, first performed by Christiaan Barnard and his team of thirty people in an operation that lasted for nine hours at Groote Schuur Hospital in Cape Town in 1967.
Understanding transplant immunology and the underlying mechanisms of acute and chronic rejection led to groundbreaking discoveries in this area (Billingham et al. 1951; Calne 1963). The identification of an immunosuppressant drug called cyclosporine, derived from soil fungus (approved by FDA 1983), was a major breakthrough for the development of organ transplantation as a standard clinical procedure for the treatment of end-stage diseases (Borel et al. 1976).

**Indications and candidate characteristics**

Worldwide, the estimated amount of people suffering from end-stage chronic kidney disease (CKD) is 1.7 million, and the number is still increasing since the rising prevalence of type 2 diabetes mellitus and diabetic nephropathy (Bendorf et al. 2013). Similarly, end-stage heart failure (HF) has reached epidemic proportions affecting up to 5.8 million people in USA and over 23 million worldwide (Liu and Eisen 2013). Thus, waiting lists for kidney transplantation have nearly doubled between 1999 and 2008, with more people dying each day as a transplant candidate (Axelrod et al. 2010).

Based on data from 109 countries, around 78,000 renal and 5,900 cardiac transplantations were performed worldwide in 2012 (The Global Observatory on Donation and Transplantation, produced by the WHO-ONT collaboration), of which ca 4100 cardiac transplantations were reported to the registry of the International Society for Heart and Lung Transplantation (ISHLT) (Lund et al. 2013). Worldwide, majority of renal transplants were from deceased donor origin (The Global Observatory on Donation and Transplantation, produced by the WHO-ONT collaboration) (Axelrod et al. 2010).

Primary indications for kidney transplantations were diabetes, glomerulonephritis, hypertension, polycystic kidney disease, structural reasons, pyelonephritis and renovascular disease (Matas et al. 2013). For heart transplantations, leading indications were ischemic and non-ischemic cardiomyopathy, coronary artery disease, congenital and valvular heart disease or retransplantation (Colvin-Adams et al. 2013) (Table 2).

**Table 2.** Transplant recipient characteristics (primary cause of disease), modified from the OPTN/SRTR 2011 Annual Data Report.

<table>
<thead>
<tr>
<th>Kidney transplant recipients</th>
<th>Heart transplant recipients</th>
</tr>
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<tbody>
<tr>
<td><strong>Pediatric</strong></td>
<td><strong>Pediatric</strong></td>
</tr>
<tr>
<td>Structural</td>
<td>Congenital defect</td>
</tr>
<tr>
<td>Pediatric</td>
<td>Adult</td>
</tr>
<tr>
<td>Focal segmental glomerulosclerosis (FSGS)</td>
<td>Dilated myopathy (idiopathic/myocarditis)</td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>Restrictive myopathy</td>
</tr>
<tr>
<td>Other</td>
<td>Other</td>
</tr>
<tr>
<td>Adult</td>
<td>Adult</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Cardiomyopathy</td>
</tr>
<tr>
<td>Hypertension</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>Congenital disease</td>
</tr>
<tr>
<td>Cystic kidney disease</td>
<td>Valvular disease</td>
</tr>
<tr>
<td>Other</td>
<td>Other</td>
</tr>
</tbody>
</table>

14
Brain death

The brain is covered by a rigid skull. Vasogenic or cytotoxic edema may increase intracranial pressure (ICP) over mean arterial pressure (MAP) resulting in non-existent cerebral perfusion pressure (CPP) and leading to irreversible loss of all brain and brainstem activity defined as brain death (The honorary secretary of the Conference of Medical Royal Colleges and their Faculties 1976; The Quality Standards Subcommittee of the American Academy of Neurology 1995; Wijdicks et al. 2010; Nakagawa et al. 2011). Thus, neurocritical care patients are typically treated with osmotic diuretics and vasopressor support (Rosner et al. 1995; Qureshi et al. 1999; Kroppenstedt et al. 2003; Kerwin et al. 2009; Sookplung et al. 2011). Mild hypothermia might be included not only to reduce metabolic activity and oxygen demand, but also to increase CPP due to lowered brain edema and ICP in subacute phase, improving survival and neurological recovery (Gal et al. 2002; Polderman et al. 2002). Cerebral protective strategy may, however, burden peripheral organs via electrolytic disbalance, impaired perfusion and arrhythmia susceptibility (Arbour 2005; Mascia et al. 2009; Dictus et al. 2009; Bohman and Schuster 2013) (Table 3). Events preceding brain death, like traumatic brain injury and subarachnoid (SAH) or intracerebral haemorrhage (ICH) aggravate a systematic inflammatory response (Yoshimoto et al. 2001; Dhar and Diringer 2008). Animal and human studies reveal that brain death elicits tremendous changes in stress hormone levels, sympathetic nervous activity, systemic blood pressure and secretion of proinflammatory cytokines and procoagulant factors. Further, the combination of primary cause and consequent brain death results in impaired haemodynamics, metabolic switch to anaerobic state, increased apoptosis and endothelial activation of peripheral organs (Chen et al. 1996; Herijgers et al. 1996; van der Hoeven et al. 1999; Chiarl et al. 2000a; Stangl et al. 2001; Szabo et al. 2002; Nijboer et al. 2004; Lisman et al. 2011). All of these events can be linked with post-transplant allograft dysfunction and alloreactivity (Birks et al. 2000b; Lopau et al. 2000; Murugan et al. 2008).

Table 3. Adverse effects of cerebral protective strategy on neurocritical care patient peripheral organ function

<table>
<thead>
<tr>
<th>Cerebral protective strategy of neurocritical care patients</th>
<th>Adverse effects on cadaver donor peripheral organ function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osmotic diuresis for reducing cerebral edema</td>
<td>Hyperosmolality, hypovolemia</td>
</tr>
<tr>
<td>Vasopressor support for maintaining cerebral perfusion pressure 60-70mmHg</td>
<td>Vascular resistance, impaired organ perfusion, lactic acidosis</td>
</tr>
<tr>
<td>Mild hypothermia (33-34°C) for protection against focal cerebral ischemia</td>
<td>Arrhythmia, reduced myocardial contractility due to decreased Ca²⁺ sensitivity</td>
</tr>
</tbody>
</table>

Donor management

The steadily declining number of brain dead organ donors due to the improvement in prevention and treatment of traumatic brain injury and intracranial bleeding (SAH and ICH) (Kompanje et al. 2006) with the concurrently increasing number of heart and kidney recipient candidates has drawn the focus on the efforts to identify potential donors (Gortmaker et al. 1996; Sheehy et al. 2003). In addition, there is rising interest in the use of donors with extended criteria or use of cardiac death/non-heartbeating donors for the expansion of the donor pool (Tuttle-Newhall et al. 2009). The heart is especially susceptible to donor induced injuries as demonstrated by low utilization proportion and fairly poor long-term survival (Smits et al. 2012; Samsky et al. 2013). Based on these aspects, management of suboptimal organ donors has received increasing attention in the field of transplant research (Smith 2004; Wood et al. 2004; Arbour 2005).
A pioneering experimental study screening potential donor treatment has laid basis on the clinical use of high-dose methylprednisolone (MP) (Soots and Hayry 1978). Complementary animal studies have confirmed the advantage of donor MP treatment in order to enhance myocardial viability and contractile recovery resulting in faster weaning from inotropes (Toledo-Pereyra and Jara 1979; Segel et al. 1997; Lyons et al. 2005). The guidelines for aggressive donor management recommend the use of hormonal resuscitation/replacement (3HR) involving vasopressin infusion and triiodothyronine or l-thyroxine (Novitzky et al. 1988; Novitzky et al. 1990; Jeevanandam et al. 1994; Jeevanandam 1997; Salim et al. 2001; Salim et al. 2007) in combination with MP bolus to induce graft utilization and quality (Rosengard et al. 2002; Zaroff et al. 2002; Rosendale et al. 2003a, 2003b). However, some studies have challenged the benefit of 3HR therapy (Randell and Hockerstedt 1992; Goarin et al. 1996; Perez-Blanco et al. 2005; Venkateswaran et al. 2009b; Venkateswaran et al. 2009a; Macdonald et al. 2012).

Organ preservation

Successful organ preservation is based on optimal temperature, the use of modern cardioplegia and preservation solutions and acceptable ischemia time. Additionally, controlled restoration of blood flow is crucial as, paradoxically, reperfusion generates an injury that greatly exceeds the injury induced by ischemia alone (Hearse 1977; Billingham et al. 1980). There is renewed interest in mechanical perfusion, supporting metabolic activity by delivering oxygen and nutrients to the graft and providing microvascular wall shear.

Cold cardioplegia, to ensure repolarization (electromechanical silence; asystole) and minimal metabolic demand, is usually based on high potassium concentrations of cold crystalloid solutions. Euro-Collins preservation solution prolonged safe cold preservation time of kidney transplants to 36-48 hours, making tissue matching, long-distance retrieval and reduction of emergency surgery possible (Jamart and Lambotte 1983). The University of Wisconsin (UW) solution, mimicking intracellular-like consistency, was the first transplant-designed preservation solution. UW solution contains high energy phosphate precursors, H+ buffering capacity, antioxidant properties, and hydroxyethyl starch (HES) containing colloids to prevent hypothermically induced cell and intracellular space swelling (edema). The UW solution soon displaced the Euro-Collins solution, due to superior cold storage properties both in kidney and heart transplants (Ploeg 1990; Ploeg et al. 1992; Stringham et al. 1998; George et al. 2011). Despite improvements in preservation solutions, especially the heart is vulnerable to hypothermic static cold preservation, cold ischemia time being the main risk factor for poor outcome (Tanaka et al. 2005). Generally, static cold storage of heart transplants should not exceed 4 hours (Marasco et al. 2005), whereas novel preservation techniques where the heart is continuously perfused with a normothermic blood may start a new era in cardiac preservation. Current median organ preservation times are 2-3 hours for cardiac and 12-21 hours for kidney transplants (OPTN/SRTR 2011 Annual Data Report: Heart and kidney). Mechanical continuos perfusion of donor hearts appears a promising turning point to answer perpetual donor shortage and the ever-increasing demand of use of donors with extended criteria, allowing tissue matching and organ sharing in heart transplants (Rosenbaum et al. 2008; Yang et al. 2013). Similarly, pulsatile machine perfusion of kidneys is associated with improved early and long-term renal function compared to kidneys subjected to static cold storage (Polyak et al. 2000; Bathini et al. 2013).
Primary graft dysfunction and delayed graft function

Primary graft dysfunction (PGD) is a devastating complication of the immediate post-operative period after heart transplantation. PGD is usually manifested by severe right or biventricular failure, leading on early institution of mechanical circulatory support (Marasco et al. 2005). PGD accounts nearly up to a quarter of heart transplants, and is the leading cause of early heart mortality after transplantation, exceeding even 50% in cases of PGD (Cosio Carmena et al. 2013). However, the 1-month mortality due to PGD differs 10-fold in previous studies, so does the practice in circulatory support between the centers/units [extracorporeal membrane oxygenation (ECMO), intra-aortic balloon pump (IABP), total artificial heart (TAH) or ventricular assist device (VAD)] (Cosio Carmena et al. 2013). The high amount of donor inotrope support, graft ischemia time exceeding 4 hours and high donor age are the major risk factors for PGD (Marasco et al. 2005; Santise et al. 2009; D’Ancona et al. 2010). In particular, in heart transplants from the donors with extended criteria, PGD complicates immediate post-operative recovery and discharge from the hospital (Iyer et al. 2011).

Delayed graft function (DGF) is a multifactorial acute renal failure after kidney transplantation. DGF occurs more frequently among deceased donors affecting still 60% of kidney transplants (Szwarc et al. 2005; Bronzatto et al. 2009). DGF features clinically oliguria and histologically reversible acute tubular necrosis (ATN). Prolonged cold ischemia time is associated with higher incidence of DGF (McLaren et al. 1999; Kayler et al. 2011; van der Vliet and Warle 2013). Even though DGF is reversible, it may predispose the graft to an increased risk for acute rejection episode and reduced graft function and affect the long-term survival (Ojo et al. 1997; Yarlagadda et al. 2009; Eid et al. 2013; Raimundo et al. 2013).

PGD and DGF may multiply the total costs of transplantations due to increases in inotropic and mechanical circulatory support after heart transplantation and need for renal replacement therapy after of kidney transplantation leading to prolonged hospitalization of the recipients (Marasco et al. 2007; Schnitzler et al. 2011; Gheorghian et al. 2012). Thus, treatments to minimize detrimental effects of prolonged cold ischemia time impoving early post-operative graft function are also a major economical issue.

Allorecognition and antigen presentation

During brain death, organ procurement and preservation, and restoration of blood flow, an allograft faces multiple non-immunological injuries that potentiate allograft immunogenecity. Released by the allograft injury, damage-associated molecular patterns (DAMPs) - ligands for Toll-like receptors (TLRs) - are believed to be crucial in triggering innate and subsequent adaptive immune responses (Mbithi et al. 1991; Matzinger 1994; Medzhitov et al. 1997) (Figure 2).

In the allogenic transplantation setting, according to the self-nonself and danger models, cellular damage during organ transplantation together with direct/indirect antigen presentation are required for the activation of cellular and humoral effector mechanisms aiming to destruct a foreign body (Janeway 1992; Medzhitov and Janeway 1997). In direct antigen presentation, CD4+ or CD8+ T cells of the recipient recognize allogenic major histocompatibility complex (MHC class I and II) molecules (non-self antigens of the graft) by donor “passenger” antigen-presenting cells (APC; mainly dendritic cells). Furthermore, the presence of donor “passenger” T cells may lead to direct activation of recipient autoreactive B cells (Win et al. 2009). Indirect antigen presentation requires the activation of the recipient
APC by recognition of endogenous molecules released by tissue injury (DAMPs), expression of donor antigens on the recipient MHC molecules and co-stimulatory activity by the same cells, and antigen presentation to unprimed T cells in secondary lymphoid organs such as lymph nodes and spleen (Liu and Janeway 1992; Fangmann et al. 1992; Lenschow et al. 1996; Vella et al. 1997; Benichou et al. 1999; Pietra et al. 2000; Whitelegg and Barber 2004). Antigen presentation leads to the activation, clonal expansion and migration of primed alloreactive T cells to the allograft (classical pathway of cellular immune response). Th2 helper T cells further trigger B cells to participate in allograft rejection by secreting antibodies directed to donor antigens (humoral immune response) (Terasaki 2003; Montgomery et al. 2004; Dragun 2008). Interestingly, indirect antigen presentation was reported to occur also independently of the secondary lymphoid organs, in the transplanted allogenic organ (tertiary lymphoid organ) (Zhou et al. 2003; Nasr et al. 2007). On the other hand, the involvement of anti-inflammatory regulatory T cells, alternatively activated (M2) macrophages and certain subpopulations of dendritic cells may counterbalance immune activation and promote tolerance for the graft (Banchereau and Steinman 1998; Nishimura et al. 2004; Tiemessen et al. 2007; Swirski et al. 2009; Wood et al. 2011; Manicassamy and Pulendran 2011).

Figure 2. Simplified presentation of how donor brain death and transplant ischemia/reperfusion injury result in adaptive immune activation.

Acute and chronic allograft rejection

Classifications of renal and cardiac allograft rejections are based on histological analysis of allograft biopsy (Billingham et al. 1990; Solez et al. 1993). Current immunosuppressive medication has markedly reduced the rate of acute rejections both in kidney and heart allografts, prolonged ischemia time and MHC-mismatch being the major risk factors for acute rejection. Acute rejections may occur between the first week and several months after the transplantation. Acute rejections are caused either by T cell dependent acute cellular rejection or B cell dependent acute antibody-mediated rejection and may decline allograft survival (Joseph et al. 2001). Despite impressive improvement in short-term graft survival, the management of chronic rejection has improved only little (Pascual et al. 2002; Meier-Kriesche et al. 2004). As compared to acute rejection, chronic rejection comprises of different immunological nature (Hayry 1996). Chronic rejection is a response to ongoing, low-grade injuries to the allograft EC (Hayry 1996). Chronic inflammatory and fibroproliferative processes are common manifestanons of chronic rejection in all transplanted organs. Chronically rejecting renal allografts develop arterial intimal thickening, glomerular sclerosis and tubular atrophy, while cardiac allografts show diffuse cardiac allograft vasculopathy and fibrosis. Chronic allograft dysfunction as a consequence of chronic rejection is the main reason for the poor long-term survival of heart and kidney allografts. Adaptive and humoral
immune responses may link ischemia-reperfusion injury to the progression of chronic rejection and reduced long-term survival (Syrrjala et al. 2010; Fuquay et al. 2013).

**Immunosuppressive medication**

The introduction of glucocorticoids to prevent acute rejections by suppressing immune responses laid the basis on the success in allogenic transplantation (Starzl et al. 1964; Hume et al. 1964; Barnard 1969). Co-morbidities of chronic steroid use became widely recognized including high susceptibility to infections, metabolic (weight gain, glucose intolerance), cardiovascular (hypertension, hyperlipidemia) and skeletal (osteopenia, impaired growth) disorders among others (Siegel et al. 1972; Park et al. 1984; Citterio 2001). The introduction of calcineurin inhibitor (CNI) cyclosporin A (CsA) revolutionized organ transplantation by the dramatic improvement in the outcome of transplant recipients (Kolata 1983). CNI exposure may result in acute and chronic nephrotoxicity (Calne et al. 1978; Nankivell et al. 2003; Naesens et al. 2009). Chronic nephrotoxicity being, however, overstated against the risk of rejections in CNI dose reduction/avoidance at the present immunosuppressive regimen repertoire (Gaston 2009; Issa et al. 2013). Indisputably, introduction of these second generation immunosuppressive drugs that more selectively target T and B cell responses, has enabled steroid minimization/weaning in maintenance and acute rejection immunosuppression by a marked reduction of steroid-related unwanted side effects both in kidney and heart transplant recipients (Ahsan et al. 1999; Vincenti et al. 2003; ter Meulen et al. 2004; Rostaing et al. 2005; Pelletier et al. 2006; Teuteberg et al. 2008).

New immunosuppressive drugs have enabled a more complex choice of immunosuppressive regimen combinations. The distinctive immunosuppressive drug use between kidney and cardiac transplant recipients reflects the different efficacy, and systemic and organ specific toxicity profiles of current immunosuppressive regimens (Pirsch et al. 1997; Jain et al. 2000; Heisel et al. 2004; Chapman 2011; Almeida et al. 2013). Over the last years, anti-interleukin-2 receptor or thymoglobulin induction therapy with tacrolimus and mycophenolate mofetil based maintenance therapy has become the most commonly used combination for solid-organ transplant recipients both in kidney and heart transplant recipients (Table 4).

<table>
<thead>
<tr>
<th>Table 4. Prevalence and the mechanisms of action of immunosuppressive drugs prescribed to kidney and cardiac transplant recipients at the time of transplantation (and 1-year post-Tx), modified from the OPTN/SRTR 2011 Annual Data Report.</th>
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</thead>
<tbody>
<tr>
<td><strong>Immunosuppression</strong></td>
<td><strong>Kidney transplant recipients</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pediatric</td>
<td>Adult</td>
<td>Pediatric</td>
<td>Adult</td>
</tr>
<tr>
<td><strong>Maintenance therapy</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Tacrolimus</td>
<td>94.0</td>
<td>89.7</td>
<td>83.2</td>
<td>85.3</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>1.6</td>
<td>3.9</td>
<td>15.2</td>
<td>9.5</td>
</tr>
<tr>
<td>Mycophenolate Mofetil/Sodium</td>
<td>92.6</td>
<td>91.2</td>
<td>90.0</td>
<td>91.9</td>
</tr>
<tr>
<td>Azathioprine</td>
<td>1.2</td>
<td>0.4</td>
<td>6.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>63.8 (62.5)</td>
<td>66.2 (65.0)</td>
<td>68.8 (66.1)</td>
<td>89.2 (87.2)</td>
</tr>
<tr>
<td>Sirolimus (and Everolimus)</td>
<td>0.3 (5.9)</td>
<td>3.2 (5.9)</td>
<td>1.6 (1.2)</td>
<td>0.6 (8.9)</td>
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<tr>
<td><strong>Induction therapy</strong></td>
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<tr>
<td>Anti-thymocyte globulin</td>
<td>48.9</td>
<td>62.3</td>
<td>48.0</td>
<td>19.7</td>
</tr>
<tr>
<td>Basiliximab/Dacizumab</td>
<td>35.0</td>
<td>24.6</td>
<td>25.7</td>
<td>26.5</td>
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<tr>
<td><strong>Group</strong></td>
<td></td>
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<tr>
<td>Calcineurin inhibitor (Tacrolimus, Cyclosporine)</td>
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<tr>
<td>Anti-metabolite (Mycophenolate Mofetil/Sodium, Azathioprine)</td>
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<tr>
<td>Corticosteroid (Methylprednisolone etc.)</td>
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<tr>
<td>mTOR inhibitor (Sirolimus/Everolimus)</td>
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<tr>
<td>Anti-thymocyte globulin (Thymoglobulin/ATGAM)</td>
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<tr>
<td>IL2-R mAb (Basiliximab/Dacizumab)</td>
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<tr>
<td><strong>Mechanism of action</strong></td>
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<td>prevents interleukin-2 transcription through nuclear factor prevents DNA synthesis through de novo purine synthesis prevents protein synthesis of pro-inflammatory cytokines, hyaluronic acid prevents interleukin-2 responses through mTOR complex 1 monoclonal rabbit/horse T-cell antibody prevents interleukin-2 receptor activity through the alpha-chain (CD25)</td>
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<tr>
<td><strong>Immunosuppressive target</strong></td>
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<tr>
<td>T-cell activation</td>
<td></td>
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<tr>
<td>T- and B-cell proliferation</td>
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<tr>
<td>Inflammatory responses</td>
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<tr>
<td>Circulating T-cell number</td>
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<tr>
<td>T-cell activation</td>
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</table>
Outcomes

The 1-year survival rates of heart and kidney transplants have substantially improved due to advancements in immunosuppressive medication, monitoring and management of acute rejections, and patient care (Johnson et al. 2010; Axelrod et al. 2010). Currently, the 1-year survival rate for cardiac allografts was 91% and that of kidney allografts 96.8% and 91.6% of living and deceased donors, respectively (OPTN/SRTR 2011 Annual Data Report: Heart and kidney). Despite, for the past 20 years survival beyond the first year after transplantation has remained relatively constant (Lodhi et al. 2011; Stehlik et al. 2012). Cardiac allograft vasculopathy and side-effects of immunosuppressive medication such as cardiovascular diseases, infections, and malignancies are the main causes of allograft loss and death 1 year after transplantation. (Colvin-Adams et al. 2013; Matas et al. 2013). At 5 years, cardiac allograft vasculopathy and late graft dysfunction account for 32%, malignancies for 23%, and infections for 10% of deaths of heart transplant recipients. Similarly of kidney transplant recipients with a specified cause of death, 21% died due to malignancies, 21% as a consequence of cardiac disease, and 16% for infections. Adjusted 5-year patient survival rates were 75% for heart transplant recipients and 85% and 74%, respectively, for kidney transplant recipients of living and cadaveric donor kidney (OPTN/SRTR 2011 Annual Data Report: Heart and kidney)(Colvin-Adams et al. 2013; Matas et al. 2013). Half-lives were 12.5 years for cardiac transplant recipients, and 13.8 and 9.7 years for kidney transplant recipients of living and cadaveric donor kidney, respectively (OPTN/SRTR 2011 Annual Data Report: Heart and kidney). Table 5 summarizes the adjusted 3-month, 1-, 3-, 5- and 10-year graft survival rates of heart and kidney transplants.

<table>
<thead>
<tr>
<th>Time (Tx year)</th>
<th>Heart transplantation</th>
<th>Kidney transplantation living / deceased donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-month (2010)</td>
<td>94.4</td>
<td>98.6 / 96.2</td>
</tr>
<tr>
<td>1-year (2010)</td>
<td>91.4</td>
<td>97.0 / 92.9</td>
</tr>
<tr>
<td>3-year (2008)</td>
<td>81.8</td>
<td>91.8 / 84.4</td>
</tr>
<tr>
<td>5-year (2006)</td>
<td>74.8</td>
<td>84.7 / 73.7</td>
</tr>
<tr>
<td>10-year (2001)</td>
<td>56.5</td>
<td>61.1 / 46.9</td>
</tr>
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</table>

Microvascular injury in allograft ischemia-reperfusion and chronic rejection

Normally, the adult microvascular network is in quiescent state. Microvascular endothelial cells (ECs) form barrier integrity avoiding leukocyte adhesion and thrombogenicity, and the surrounding pericytes are relaxed maintaining uninterrupted blood flow. In addition, endothelial cells interplay with cardiomyocytes regulating cardiomyocyte contractile function and survival (Narmoneva et al. 2004; Xaymardan et al. 2004; Hsieh et al. 2006b; Hsieh et al. 2006a). In organ transplantation, Tx-IRI induces microvascular dysfunction via a pro-inflammatory cytokine storm, vascular permeability and perfusion disturbances, leukocyte adhesivity and thrombosis, and vasoconstriction. Microvascular dysfunction also predisposes the allograft to intimal smooth muscle cell proliferation (Hayry 1996; Lemstrom et al. 2002). Ultimately, endothelial cell dysfunction in transplants predicts the formation of chronic allograft vasculopathy and impaired long-term outcome (Hollenberg et al. 2001; Marti et al. 2001; Tona et al. 2006; Kubrich et al. 2008). Proinflammatory endothelium from old donors
seems to be especially vulnerable to Tx-IRI exposing allografts to increased immunogenicity and early and late adverse events (de Fijter et al. 2001; Korkmaz et al. 2013). Lymphatic endothelial cells are also crucial in immune surveillance as their activation regulates leukocyte traffic and rejection in allografts (Nykanen et al. 2010). Overall, vasculoprotection targeted therapy in Tx-IRI may represent an important strategy against adaptive immune response in the development of acute and chronic allograft dysfunction (Basile 2007).

**Vascular wall shear stress**

The vascular wall is continuously subjected to dynamic mechanical forces of blood flow in the form of pulsatile vertical pressure and laminar shear stress and oscillatory shear forces created by the heart beat. Shear stress and cyclic stretch play an important role in endothelial functions by inducing secretion of shear stress dependent vascular stabilizing, anti-inflammatory, anti-apoptotic, anti-thrombotic transcription factor KLF-2 and vasculoprotective down-stream genes eNOS and HO-1 (SenBanerjee et al. 2004; Lin et al. 2005; Huddleson et al. 2005; Fledderus et al. 2007; Ali et al. 2009; Boon et al. 2010; van Agtmaal et al. 2012). Endothelial shear stress also inhibits the expression of pro-inflammatory and vascular destabilizing transcription factors NF-kB and Rho GTPases (Chiu et al. 2005; Tzima 2006; Wang et al. 2007), and leukocyte recruitment and vascular smooth muscle cell (vSMC) proliferation (Ando et al. 1994; Sheikh et al. 2003; Wang et al. 2006; Matharu et al. 2008).

Lack of vascular wall haemodynamic forces, as during static cold preservation of organ transplants, predisposes allografts to cytoskeletal rearrangement and barrier disruption (Morita et al. 1993; Cheng et al. 2007). Strategies to improve vascular homeostasis during preservation and organ transportation involve mechanical perfusion (discussed above in “Organ Preservation” on pages 16-17) or pharmacological interventions stabilizing vascular homeostasis (EC viability) by affecting the expression and activity of blood flow-regulated genes (discussed later in “HMG-CoA reductase inhibitors” on pages 26-29).

**Leakage and perfusion defects (the no-reflow phenomenon)**

In quiescent vascular wall, endothelium forms an integral barrier against vascular leakage and leukocyte extravasation and pericytes and vSMCs are in relaxed state responding for vascular patency. During allograft procurement and acute hypoxia, activation (phosphorylation) of Rho GTPases, myosin light chain (MLC)2 and ezrin-radixin-moesin (ERM) proteins re-organize actin-myosin II cytoskeleton of vascular endothelial cells and perivascular pericytes and SMCs (Chrzanowska-Wodnicka and Burridge 1996; Amano et al. 1996; Hall 1998; Wojciak-Stothard et al. 2005). Hypoxia-induced rapid actin stress-fiber formation mechanistically causes endothelial adherense and tight junction opening and sustained pericyte/vSMC contraction (Kutcher and Herman 2009; Yemisci et al. 2009). Hypoxic stress-fiber formation as well as hyperoxic oxidative stress in re-oxygenation further trigger vascular destabilizing angiopoietin (Ang)-2 and constrictive endothelin (ET)-1 release from EC Weibel-Palade bodies and impair endothelial NO (eNOS) expression and its autovasodilatory responses (Shyu et al. 2003; Bhandari et al. 2006; de Vries et al. 2013).
Together, these result in vascular permeability and deteriorate tissue perfusion (the no-reflow phenomenon). RhoA-Rho kinase pathway activity has turned out to be a notable therapeutic target in the prevention of microvascular injury during IRI (Prakash et al. 2008; Li et al. 2011; Kentrup et al. 2011; Shi and Wei 2013).

Endothelial cell interactions

Communication between endothelium and cardiomyocytes regulates cardiomyocyte proliferation, organization, maturation, and function (Brutsaert 2003). Under *in vitro* coculture conditions cardiomyocytes exclusively survive in EC closeness (resembling the *in vivo* situation where cardiomyocytes are in close spatial relationship with endothelial cells of the capillary network) (Narmoneva et al. 2004; Davis et al. 2005) (Figure 3). Furthermore, endothelial cells may induce myocyte hypertrophy as a response to persistent hemodynamic stress (pressure overload; high blood pressure) (Tirziu and Simons 2008). Discovery of endothelial pro-survival effect on cardiomyocytes has challenged the idea of capillary endothelial cells being simply suppliers of oxygenated and nutritious blood and suggests a more complex role in cardiac health and viability.

Endothelial cell derived angiogenic growth factor platelet derived growth factor (PDGF)-BB directly preserves cardiomyocyte survival and systolic function via PI3K/Akt-pathway in myocardial IRI and infarction models (Hsieh et al. 2006a; Hsieh et al. 2006b). Conversely, the fact that PDGF-B-chain inhibition increased arterial SMC apoptosis, points out that targeting angiogenic growth factors could be used to induce neointimal regression aiming to reverse hyperplasia lesions (Leppanen et al. 2000; Schermuly et al. 2005). This in accordance with the previous findings in experimental chronic rejection models, where cardiac allograft vasculopathy was minimized by blocking PDGF and vascular endothelial growth factor (VEGF) receptor activity (Lemstrom et al. 2002; Sihvola et al. 2003; Nykanen et al. 2005).

*Figure 3.* (A) Heart is more than just a bunch of cardiomyocytes. Roughly, myocardium consist of an equal amount of cardiomyocytes and capillaries; nearly 2000 per mm² each. Inset, myocyte membranes (red), capillaries (green) and DAPI+ nuclei (blue) reprinted from Jaba IM et al., J Clin Invest., 2013 Apr 1;123(4):1718-31 with the permission from the American Society for Clinical Investigation (left) and Hsieh PC et al., Annu Rev Physiol., 2006;68:51-66 with the permission of Annual Reviews (right). (B) Over 95% of the
cultured neonatal cardiomyocytes (red) choose to migrate to or survive in proximity with microvascular endothelial cells (green) from Narmoneva DA et al., Circulation, 2004 Aug 24;110(8):962-8 with the permission from the American Heart Association and Lippincott Williams and Wilkins (LWW).

**Endothelial-to-mesenchymal transition (fibroproliferation)**

Interstitial fibrosis, a characteristic feature of chronic rejection in the allografts, accounts for accumulation of fibroblasts in the interstitium with phenotypic appearance of myofibroblasts and extensive deposition of extracellular matrix. Interstitial fibrosis replaces the functional parenchyma and contributes to the progressive loss of its function. Interstitial fibrosis may arise from proliferation of resident fibroblast, the migration and differentiation from bone marrow-derived cells and vascular pericytes, and the epithelial-to-mesenchymal transition (EMT) (Iwano et al. 2002; Broekema et al. 2007; Lin et al. 2008). Currently, there is no specific treatment to control interstitial fibrosis.

Mesenchymal transition is critical in the embryonic development of heart and kidney (Hay and Zuk 1995; Eisenberg and Markwald 1995). Recently, endothelial-to-mesenchymal transition (EndMT) was discovered as a remarkable contributor to interstitial fibrosis of the transplant as up to half of fibroblasts co-express the endothelial marker (Zeisberg et al. 2007; Zeisberg et al. 2008). Similar underlying mechanisms that evoke vascular dysfunction relate to the fibroproliferation of allografts (Babu et al. 2007). Both pathophysiological circumstances involve endothelial cytoskeletal rearrangement, loss of inter-endothelial integrity and detachment from supporting basal lamina. Hypoxia causes HIF-1α activation, which participates in MLC2 phosphorylation and endothelial barrier disruption (Qi et al. 2011; Wang et al. 2013), but also promotes mesenchymal transition (Higgins et al. 2007). Moreover, loss of vascular inter-endothelial junction VE-cadherin in ischemia-reperfusion injury promotes peripheral blood mononuclear cell infiltration, pro-fibrotic TGF-β1 expression and EndMT (Sutton et al. 2003; Li et al. 2009; Ghosh et al. 2010; Vockel and Vestweber 2013).

**Vascular remodeling**

Allograft vasculopathy, a manifestation of chronic rejection, is one of the main reasons that limit long-term survival of renal and cardiac allografts. It is characterized by structural changes - atypical from atherosclerotic plaques - concentric intimal proliferation and vascular occlusion, and is detectable in half of cardiac transplants by 5 years after transplantation (Gao et al. 1989; St Goar et al. 1992; Rickenbacher et al. 1995). The pathogenesis of allograft vasculopathy is not completely understood, but nonimmunological innate vascular injury together with chronic alloimmune dependent pro-inflammatory milieu are believed to trigger SMC activation and fibroproliferative response in the allograft. This includes upregulation of chemokines and pro-inflammatory cytokines, mononuclear cell infiltration and the recruitment, proliferation and matrix synthesis of vascular SMC in the intima of injured arteries, and occlusion of the vascular lumen by neointimal formation (Rahmani et al. 2006; Schober 2008). Vascular SMC are traditionally believed to derive from the local vessel wall; however, in some instances and to some extent, at least in animal models recipient-derived hematopoietic progenitor cells may also contribute to the neointimal formation (Hillebrands et al. 2001; Li et al. 2001; Shimizu et al. 2001; Hu et al. 2002).
Allograft EC forms the interface/barrier with recipient’s immune system and is the primary target of recipient immunologic defences (Pober et al. 1984; Salomon et al. 1991). Endothelial function in the allografts declines over time (Treasure et al. 1992). Endothelial dysfunction is suggested to play a central role in the progression of allograft vasculopathy and to predict independently the outcome of the patient (Davis et al. 1996; Schachinger et al. 2000; Hillebrands et al. 2000; Marti et al. 2001; Hollenberg et al. 2001; Kubrich et al. 2008). Growth factors play a crucial role in the physiological healing processes, e.g., in endothelial repair, angiogenesis and scar formation. According to the response-to injury theory, however, persistent vascular endothelial injury of transplanted organs due to chronic alloimmune responses results in an uncontrolled repair process by prolonged growth factor expression leading to a pathological fibroproliferative disease (Lemstrom et al. 1995). Preventing multifactorial allograft vasculopathy necessitates the development of pharmacological compounds capable of prophylaxis of EC dysfunction during transplantation procedure, rather than therapy directed to fight neointimal formation by inhibiting SMC proliferation or pre-existing lesions by inducing SMC apoptosis (Lemstrom et al. 1995).

HMG-CoA reductase inhibitors

Statins are drugs that inhibit the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and lower blood cholesterol levels in patients with hyperlipidemia (Figure 4). Based on the solid evidence in multiple large clinical trials of its effectiveness and safety, statins are recommended for primary and secondary prevention of cardiovascular diseases for a wide range of high-risk patients, regardless of their initial cholesterol levels (the Scandinavian Simvastatin Survival Study 1994; Shepherd et al. 1995; (LIPID) Study Group 1998; the LIPID trial follow-up 2002; Cannon et al. 2004; LaRosa et al. 2005; Amarenco et al. 2006).

![Figure 4. Simplified diagram of lipid lowering and pleiotropic mechanisms of action of statins.](image-url)
Pleiotropic effects

In addition to lipid lowering ability, statins have a plethora of beneficial pleiotropic effects such as improvement of vascular EC and pericyte function, reduction of inflammation, and inhibition of tissue remodeling independent of lipid lowering (Endres et al. 1998; Koh 2000; Wilson et al. 2001; Wilson et al. 2002; Bonetti et al. 2002; Zhao et al. 2006). These drugs seem to have also anti-oxidative, -anti-coagulant and anti-thrombotic effects (Liao and Laufs 2005; Ali et al. 2007). Direct anti-inflammatory effects may be partially explained by HMG-CoA independent lymphocyte function-associated antigen (LFA)-1 inhibition on T cells (Weitz-Schmidt et al. 2001), important for lymphocyte adhesion and activation (Schramm et al. 2007; Takahashi et al. 2007). Statins maintain vascular homeostasis by regulating its cytoskeletal rearrangement, growth factor release and reactivity at the site of injury (Hernandez-Perera et al. 1998; Alber et al. 2002; Undas et al. 2006; Lee et al. 2011). This involves inhibition of stress fibre formation of endothelial and perivascular supporting cells and endothelial exocytosis of VEGF and Weibel-Palade body factors via the family of Rho GTPases, and KLF2 (Takeimoto and Liao 2001; Yamakuchi et al. 2005; Ho et al. 2008; van Agtmaal et al. 2012; Hilbert et al. 2013). Inhibition of blood clotting and thrombosis may be explained both by direct and indirect mechanisms. Statins reduce endothelial and macrophage tissue factor (TF) expression and secretion of von Willebrand factor (vWF), and induce thrombomodulin expression RhoA and KLF2 -dependently (Colli et al. 1997; Szczeklik et al. 1999; Undas et al. 2001; Eto et al. 2002; Masamura et al. 2003), and have effects on platelet activation (Sanguigni et al. 2005; Pignatelli et al. 2012). Furthermore, statins contribute indirectly to hemostasis and thrombosis via reduced vascular injury and inflammation. Despite the relatively comparable lipid-lowering potency of statins, their ability to exert extrahepatic pleotropic effects differs largely due to dissimilar pharmacokinetic properties, mostly of lipophilicity qualities (Schachter 2005; Mason et al. 2005; Bonsu et al. 2013).

Pre- and postoperative treatment in cardiac and kidney ischemia-reperfusion

Pre- and early postoperative use of statins is associated with improved cardiac and kidney function after major elective surgery (Pan et al. 2004; Pasceri et al. 2004; Welten et al. 2008; Kulik and Ruel 2009; Ege et al. 2010; Billings et al. 2010; Molnar et al. 2011, , Brunelli, 2012 #568; Kuhn et al. 2013; Singh et al. 2013). Moreover, findings on the cardio- and renoprotective effects of statins are supported by acute preoperative statin administration before elective surgery in clinical trials in patients undergoing percutaneous coronary intervention (PCI) (Patti et al. 2007; Di Sciascio et al. 2009; Gibson et al. 2009; Patti et al. 2011) and coronary artery bypass grafting (CABG) (Mannacio et al. 2008; Ji et al. 2009; Antoniades et al. 2010; Sun et al. 2011; Baran et al. 2012). However, a current meta-analysis of randomized controlled trials including participants undergoing various cardiac surgical procedures challenges these conclusion by stating that preoperative statin treatment shortens the intensive care unit (ICU) and total hospital stay without affecting perioperative mortality, cardiovascular adverse events, or renal failure (Liakopoulos et al. 2012).

Based on the clinical experience, it is reasonable to administrate statins with immunosuppressive medication to reduce allograft vasculopathy and cardiovascular mortality in heart transplant recipients (Kobashigawa et al. 1995; Katzenelson and Kobashigawa 1995; Weis and von Scheidt 1997; Wenke et al. 1997; Wenke et al. 2003; Kobashigawa et al. 2005). Kidney transplant recipients benefit from statins against cardiovascular mortality (Jardine et
al. 2004; Holdaas et al. 2005; Navaneethan et al. 2009) and general mortality (Wiesbauer et al. 2008). The findings have been contradictory whether early statin use in combination with conventional immunosuppression affects the incidence and intensity of acute and multiple rejections episodes (Katznelson et al. 1996; Holdaas et al. 2001), and late graft survival in kidney transplant patients (Seron et al. 2008; Younas et al. 2010).

Organ donors do not have major cardiovascular disease nor tend to have previous medical track record of statin use. Donor treatment is a fascinating approach to prophylactically target TX-IRI induced microvascular injury and induction of allograft immunogenicity. Preclinical studies have shown that rapid direct vasculoprotective and anti-inflammatory effects of statins (van Nieuw Amerongen et al. 2000; Wei et al. 2005; Zeng et al. 2005; Geissler et al. 2006; Yasuda et al. 2006; Kircher et al. 2008; Tuomisto et al. 2008; Chen et al. 2008) could be exploited in transplantation settings by donor treatment to reduce ischemia-reperfusion in cardiac and renal allografts (Table 6). All of those studies, however, unfortunately lack data on clinically relevant major injuries caused by brain death, cold and warm ischemia, or alloimmune response, or do not fit with the clinically relevant pretreatment time windows. Thus, conclusions drawn from these studies are difficult to equal with the human transplantation setting. In the future, randomized clinical trials will hopefully answer whether vasculoprotective and anti-inflammatory properties of statins could be exploited in multiple organ donors to improve the quality of transplants and expansion of the donor pool.
Table 6. Previous literature of statin pretreatment in kidney IRI, and donor treatment in cardiac and kidney transplantation.

<table>
<thead>
<tr>
<th>Statin Type</th>
<th>kidney IRI</th>
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NITELY RH
Vascular growth factors

Ischemia-reperfusion injury (IRI) may lead to microvascular dysfunction and parenchymal injury with deleterious consequences for allografts (Salahudeen et al. 2004; Tanaka et al. 2005). Vascular growth factors have a regulatory role in these events. Vascular growth factor signaling mediates neointimal formation and exacerbates chronic rejection in cardiac allografts (Lemstrom and Koskinen 1997; Lemstrom et al. 2002), but on the other hand it may exert vascular stabilizing and anti-apoptotic actions in the allograft through endothelial-pericyte and endothelial-cardiomyocyte crosstalk. The balance between these potentially harmful and beneficial actions determines the final outcome of the allograft.

Platelet-derived growth factor in cardiac allograft acute and chronic rejection

Platelet derived growth factor (PDGF) ligands and their receptors are part of a family of vascular growth factors that guide physiological mesenchymal cell functions during embryogenesis, angiogenesis and wound healing (Ross et al. 1990a; Battegay et al. 1994; Leveen et al. 1994; Bostrom et al. 1996; Uutela et al. 2001; Uutela et al. 2004). In addition to physiological effects, PDGF has a regulatory role in several pathological conditions such as arteriosclerosis, rheumatoid and fibroproliferative diseases and tumor growth (Ross 1993; Lokker et al. 2002; Ponten et al. 2003; Ponten et al. 2005; Schermuly et al. 2005). Functional differences of PDGF ligands are mediated by their unique receptor-binding affinity, ability to bind to extracellular matrix (ECM), and activation-dependence by proteases (Raines et al. 1992; Fredriksson et al. 2004). Studies with knock-out mice suggest that in embryogenesis, PDGF-AA and -CC are the principal ligands responsible for PDGFR-α signaling, whereas PDGF-BB, and PDGF-DD to a lesser extent, for that of PDGFR-β (Li et al. 2000; Bergsten et al. 2001; Betsholtz et al. 2001; Ding et al. 2004). Divergent from homodimeric PDGF-AA and -BB, PDGF-AB has high affinity both to PDGFR-αα and -αβ, but not -ββ (Seifert et al. 1993).

In rat cardiac allografts, PDGF ligands and their receptors are significantly induced during the first postoperative week (Sack et al. 2004). Administration of PDGF-A, -C, and -D provoked pathological coronary intimal and myocardial fibroproliferation (Mancini and Evans 2000; Tuuminen et al. 2009). In heart transplantation model with minimal ischemia alloimmune activation was reduced with imatinib mesylate, a potent inhibitor of PDGF receptor activity (Sihvola et al. 1999; Buchdunger et al. 2002; Sihvola et al. 2003; Nykanen et al. 2005). Administration of imatinib mesylate also reversed advanced pulmonary vascular neointima formation and reduced carotic artery stenosis by inducing vascular smooth muscle cell (vSMC) apoptosis (Leppanen et al. 2000; Schermuly et al. 2005).

During hypoxia and metabolic compromise, PDGF-AB and -BB ligands play a pivotal role in microvascular and cardiomyocyte survival, endothelial repair, vascular stability and inhibition of inflammation, and cardiomyocyte contractile function (Kodama et al. 2001; Edelberg et al. 2002; Xaymardan et al. 2004; Langley et al. 2004; Hsieh et al. 2006a; Hsieh et al. 2006b; Zymek et al. 2006; Vantler et al. 2010; Kim et al. 2011; Fuxe et al. 2011). Blockade of PDGF receptor signaling abrogated PDGF-B-dependent survival and induced the activation of procaspase-3 and apoptosis of microvascular endothelial cells (Langley et al. 2004). Previous
clinical observations have shown that PDGF receptor inhibition is associated with heart failure (Kerkela et al. 2006; Chu et al. 2007). Furthermore, previous reports have shown that activation of survival pathways in hypoxia is PDGF-B/PDGFR-β dependent (Zhang et al. 2003). PDGF-BB pretreatment protected also neurons from toxicity-induced and lung fibroblasts from starvation-induced apoptosis (Tseng and Dichter 2005; Cartel and Post 2005). Nevertheless, prolonged upregulation of PDGF-B may contribute to fibroproliferation and neointimal formation by recruiting mesenchymal cells, and enhancing their proliferation and survival (Ross et al. 1990b; Golden et al. 1991).
AIMS OF THE STUDY

The aim of the study was to investigate the underlying molecular mechanisms and functional parameters of microvascular dysfunction in cardiac and renal allografts during ischemia-reperfusion injury. Furthermore, we aimed to target ischemia-reperfusion injury in cardiac and renal allografts with vasculoprotective simvastatin and immunomodulatory methylprednisolone using a clinically relevant donor treatment protocol.

The specific aims of the study were:

1) to characterize the effect of cold preservation and warm ischemia on microvascular dysfunction in the heart and kidney in the rat
2) to define the pharmacokinetics of simvastatin in the rat and in the human brain-dead organ donors
3) to compare simvastatin treatment of donors, recipients, or both donors and recipients in rat cardiac and renal allografts
4) to investigate the class effect of statins (HMG-CoA reductase inhibitors), the dose-response and the impact of different preservation solutions in rat cardiac allografts during IRI
5) to target the preservation, IRI, acute and chronic rejection in rat cardiac and kidney allografts by donor simvastatin treatment
6) to clarify the exact role of different cell types in mediating the protective effects in rat cardiac and kidney allografts by donor simvastatin treatment
7) to dissect the vasculoprotective mechanisms of rat donor simvastatin treatment by pharmacological inhibition and supplementation of specific signaling pathways
8) to pursue dual vasculoprotective and immunomodulatory management of rat cardiac allografts by combined donor simvastatin and methylprednisolone treatment
9) to evaluate the expression of PDGF receptors and ligands in rat cardiac allograft acute and chronic rejection
10) to study exogenous expression of PDGF ligands on rat cardiac allograft chronic rejection
MATERIALS AND METHODS

1. Experimental procedures

Specific, pathogen-free, inbred male Wistar Furth (WF, RT1u) and Dark Agouti (DA, RT1a) rats (Scanbur, Sollentuna, Sweden) weighing 300–350 g were used. The rats received regular rat food and tap water ad libitum, and were maintained on a 12-h light/dark cycle. Permission for animal experimentation (ESLH-2007-07748/Ym-23 (H)) was obtained from the State Provincial Office of Southern Finland. The animals received good 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 0-309-15400-6, revised 2011).

Heart and kidney preservation. The rats were anesthetized with inhalational isoflurane and a midline abdominal incision was performed. The heart or right kidney was removed. Hearts were subjected either to no cold ischemia and no warm ischemia, or to 4-hour cold ischemia and no warm ischemia, or to 4-hour cold ischemia and 1-hour warm ischemia. Kidneys were subjected either to no cold ischemia and no warm ischemia, or to 16-hour cold ischemia and no warm ischemia, or to 16-hour cold ischemia and 1-hour warm ischemia.

Renal artery clamping. The rats were anesthetized with inhalational isoflurane and a midline abdominal incision was performed. Either the right renal artery was clamped for 30 minutes to appreciate ischemia of the clamped right kidney as well as the remote ischemia of the non-clamped contralateral left kidney, or both renal arteries were clamped for 30 minutes for the analysis of post-ischemic renal function and kidney injury. After clamp removal, the kidneys were inspected for recovery of blood flow and the abdomen was closed. The rats were administered 1 ml of saline and 0.15 mg/kg s.c. of buprenorphinum (Temgesic 0.3 mg/mL, Schering-Plough, Kenilworth, NJ) for post-operative maintenance of fluid balance and pain relief, respectively.

Heterotopic heart transplantations. Intra-abdominal heterotopic heart transplantations were performed from specific pathogen-free fully MHC-mismatched inbred male Dark Agouti (DA, RT1av1) to male Wistar Furth (WF, RT1u) rats (Figure 5) and inbred male BALB/c (B/c, H-2d) to C57BL/6J (B6, H-2b) mice (Harlan, Horst, The Netherlands) 2-3 months of age. The donors were anesthetized with inhalational isoflurane and a midline abdominal incision was performed. After infusion of heparinized ice-cold PBS or Plegisol cardioplegia solution (Hospira, Inc., Lake Forest, Il) into the inferior vena cava of the heart donor, the vena cava and pulmonary veins were ligated with 6-0 silk and the pulmonary artery and aorta were cut 2 to 3 mm above their origin in the heart. After removal, allografts were left without hypothermic preservation or were preserved either in heparinized PBS or Plegisol cardioplegia solution at +4 °C for 0, 2 or 4 hours depending on the study model. Cardiac allograft recipients were anesthetized with isoflurane anesthesia (2-5%/l O2), and received buprenorphine 0.15 mg/kg s.c. for peri- and postoperative analgesia. A midline incision was made, and the aorta and pulmonary artery of the allograft were anastomosed to the abdominal aorta and inferior vena cava of the recipient, respectively. Warm ischemia occurring during heart transplantation was standardized to one hour. The allografts were harvested 5, 20 or 30 minutes, 6 hours, or 10, 56 or 100 days after the transplantation or in an acute rejection model.
when the allograft function was lost.

**Figure 5.** Rat heterotopic heart transplantation model in simvastatin treatment studies.

**Heterotopic kidney transplantations.** Intra-abdominal heterotopic kidney transplantations were performed from specific pathogen-free fully MHC-mismatched male Dark Agouti (DA, RT1av1) to male Wistar Furth (WF, RT1u) rats. The donor DA rats were anesthetized with inhalational isoflurane and a midline abdominal incision was performed. The donor kidney was perfused with 250 IU heparin in 5 ml ice-cold PBS and removed with a segment of aorta and vena cava. The kidney was preserved in +4°C PBS for 16 hours. The left native kidney was removed during the transplantation and the donor allograft aorta and vena cava were anastomosed end-to-side into heterotopic position of the recipient aorta and vena cava. The ureter was anastomosed end-to-end. Warm ischemia occurring during kidney transplantation was standardized to one hour. The allografts were harvested 5 minutes, 6 hours or 5 days after the transplantation.

2. Drug administration

**Donor simvastatin and methylprednisolone treatment.** Two hours before the heart and kidney preservation, induction of warm ischemia, or graft removal for transplantations the DA rats received either peroral simvastatin (0.5, 2.0 or 5.0 mg/kg; Merck Research Laboratories, Whitehouse Station, NJ), peroral pravastatin (5.0 mg/kg; P4498 pravastatin sodium salt, Sigma-Aldrich, St. Louis, MO) or intravenous methylprednisolone (15.0 mg/kg; Pfizer Inc., New York, NY) as a single dose. Simvastatin was diluted with polyethylene glycol (PEG, molecular weight 300; Sigma-Aldrich) to a concentration of 1.5 mg/ml, and the control rats received PEG vehicle p.o.

**Recipient simvastatin treatment.** The recipient rats were given peroral simvastatin (2.0 mg/kg) diluted with PEG to a concentration of 1.5 mg/ml 2 hours before reperfusion and daily thereafter (2 mg/kg/d), and the control rats received PEG vehicle daily p.o.

**Cyclosporine A.** To prevent severe episodes of acute rejection and to allow the development of an alloimmune response and moderate chronic rejection, allograft recipients in over 6-hour transplantation models excluding the acute rejection study were given cyclosporine A (CsA, Novartis, Basel, Switzerland) diluted with Intralipid (Fresenius Kabi, Bad Homburg, Germany). Carciac allograft recipients were treated with CsA 2.0 mg/kg/d s.c. for the first 7
days and 1.0 mg/kg/d thereafter, whereas the dose given to kidney allograft recipients was 1.5 mg/kg/d.

**Imatinib mesylate.** To inhibit PDGF receptor activity, protein tyrosine kinase inhibitor imatinib mesylate (Gleevec®, Novartis, Basel, Switzerland) was diluted with saline to a concentration of 10 mg/ml and administered to the rat cardiac allograft donors 6 hours before procurement and to the rat cardiac allograft recipients at the time of reperfusion and daily thereafter at a dose of 10 mg/kg i.p. Saline vehicle was given i.p. to the control cardiac allograft recipients.

**Inhibition or induction of specific signaling pathways.** To inhibit NOS activity, N-nitro-L-arginine methyl ester (L-NAME; Sigma-Aldrich) was dissolved in drinking water which was changed every other day. Donor rats received the orally active L-NAME (~50 mg/kg/day) for 4 days before allograft removal. To block HO-1 activity, zinc protoporphyrin (ZnPP; Porphyrin Products Inc., Logan, UT) was dissolved in 0.2M NaOH, adjusted to pH of 7.4, and diluted with 0.9% NaCl to a concentration of 1 mg/ml. ZnPP was administered i.p. (20 mg/kg) to donor rats 2 hours before allograft removal. To supplement RhoA GTPase prenylation pathway, geranylgeranyl pyrophosphate (GGPP; Sigma-Aldrich) at a concentration ~1 mg/ml in methanol was administered i.p. (1 mg/kg) to donor rats 2 hours before allograft removal. To block specific PDGFR-α or -β receptor activity during allograft ischemia-reperfusion donor mice were treated one day before cardiac procurement with rat IgG (Sigma-Aldrich, St. Louise, MO) 800 μg i.p. or rat anti-mouse PDGFR-α (IMC-3G3; ImClone, New York, NY) or rat anti-mouse PDGFR-β (IMC-2C5; ImClone) neutralizing antibody 800 μg i.p.

**Drug assay.** After collection the blood samples were immediately centrifuged at 2000 rpm for 10 min at +4 °C and stored at -80 °C until further analysis. The blood concentrations of simvastatin lactone and simvastatin β-hydroxy acid were measured by liquid chromatography-ionspray tandem mass spectrometry.

**Lipid analysis.** To analyze the effect of recipient simvastatin treatment on blood lipid levels, serum samples were collected during the cardiac allograft harvest at 8 weeks and stored at -20°C until further analysis. The concentration of total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) were measured using the enzymatic colorimetric test (Roche Diagnostics Cholesterol CHOD-PAP method; Cat. no. 1491458; Roche Ltd, Basel, Switzerland) and a biochemical analyzer (Hitachi Modular PP-analyzer; Hitachi Ltd., Tokyo, Japan).

**3. Kidney function and injury analysis**

**Renal function.** Serum was collected and subsequently frozen at -20°C until further analysis of serum creatinine and urea nitrogen activities.

**Albuminuria and urine flow rate.** To estimate glomerular vascular leakage 30 minutes after warm ischemia, the 24-hour trough urinary albumin waste was analyzed in metabolic cages.
for 3 consecutive days. Albumin concentrations were measured by photometry. Urine flow rate was calculated by the following formula: \( \text{UFR} = \frac{\text{UV}}{\text{T} \times \text{BW}} \), where UFR is the urine flow rate, UV is the urine volume, T is the time, and BW is the body weight of the rat.

**Neutrophil gelatinase-associated lipocalin.** As a biomarker for acute kidney injury, we used rat neutrophil gelatinase-associated lipocalin (NGAL). NGAL serum levels were estimated by ELISA using mouse monoclonal anti-NGAL antibody (ABS 039-08 from BioPorto Diagnostics A/S, Gentofte, Denmark) at 3 days after bilateral renal artery clamping or at 6 hours and 5 days after kidney transplantation.

**In Situ apoptosis detection.** The analysis of kidney allograft apoptosis was based on Terminal deoxynucleotidyl-transferase (TdT)-mediated dUTP nick end-labeling assay (TUNEL). Paraformaldehyde-fixed paraffin sections were stained with an *in situ* apoptosis detection kit (CardioTACS™, 4827-30-K, Trevigen Inc., Gaithersburg, MD). TUNEL-positive apoptotic nuclei were counted from four random fields of each quadrant of a renal cross section with 40x magnification, and the results are given as the mean number of positive cells per 1 mm².

**Acute tubular necrosis.** Tubular damage was determined by a senior pathologist from paraformaldehyde-fixed paraffin sections stained with hematoxylin and eosin and scored semiquantitatively (0 to 3) as follows: 0, no damage; 1, mild damage; 2, moderate damage; and 3, severe damage.

**4. Cardiac function and injury analysis**

**Cardiac allograft function.** Cardiac allograft function was estimated by daily abdominal palpation, and graded according to heart beat from 0 to 4: 0 minimal or no contractility (heart beat <30 bpm); 1, poor; 2, moderate; 3, impaired; and 4 normal. Allografts were removed when the grade fell to 0.

**Myocardial ischemia.** Pimonidazole hydrochloride (HP2-1000, Hypoxyprobe™-1 Kit, Chemicon International, Temecula, CA) was used to determine the extent of ischemic areas of cardiac allografts after reperfusion. Pimonidazole binds to thiol-containing proteins specifically in hypoxic cells. After cardiac allograft reperfusion, pimonidazole hydrochloride (60 mg/kg) was injected to penile vein i.v. and cardiac allografts were harvested at five minutes for immunofluorescence evaluation of the hypoxic regions using a primary fluorescein (FITC)-conjugated mouse monoclonal antibody directed against pimonidazole protein adducts and a secondary mouse anti-FITC monoclonal antibody conjugated to horseradish peroxidase (HRP).

**Cardiac troponin T.** The rat serum levels of cardiac troponin T (cTnT) were analyzed with the third generation troponin T test (Troponin T STAT, Roche Diagnostics), which shows cross-reactivity of 0.001% with TnT originating in skeletal muscle at a concentration of 2.000 ng/ml. The functional sensitivity is 0.03 μg/l and the lower detection limit 0.01 μg/l. The
cTnT was measured by electrochemiluminescence immunoassay (ECLIA) on the Elecsys 2010 immunoassay analyser (Roche Diagnostics) 6 hours after heart transplantation.

5. Histopathology

**Immunohistochemistry and immunofluorescence stainings.** Four µm thick paraffin-embedded or cryostat cross-sections were cut in series on glass slides, stained using the peroxidase ABC method (Vectastain Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA) and the reaction was revealed by 3-amino-9-ethylcarbazole (AEC, Vector Laboratories). The specimens were blocked with a 20-min incubation with 1.5% normal goat serum/phosphate-buffered saline (PBS), pH 7.40, followed by incubation with primary antibodies at optimal dilution at room temperature (RT) for 30 min (monoclonal antibodies) or at +4°C for 15 hours (polyclonal antibodies). The primary antibodies were diluted with a 0.1% bovine serum albumine/PBS solution. After washing in PBS, endogenous peroxidase activity was blocked with 10-min incubation with 0.1% hydrogen peroxidase (30%/PBS solution. With intervening washes in PBS, the specimens were further incubated with biotinylated antibodies in the PBS buffer at RT for 30 min; the avidin-biotinylated horseradish complex in the PBS buffer at RT for 30 min and the reaction was revealed by AEC. Counterstaining was performed using Mayer’s hemalum. Immunofluorescent stainings were performed using Alexa 568 red and Alexa 488 green (Promega, Madison, WI) secondary antibodies and DAPI nuclear staining (VECTASHIELD Mounting Medium with DAPI; Vector Laboratories).

Hyaluronan (HA) was stained from paraffin sections using a specific biotinylated bHABC hyaluronan binding complex, which contains the biotinylated G1 domain of aggrecan and a link protein, prepared from bovine articular cartilage in our laboratory, by avidin-biotin-peroxidase detection (Vector Laboratories; 1:200 dilution) with 0.05% 3,3′-diaminobenzidine (DAB) (Sigma Chemical Co.) as described (Tammi et al. 1994). The specificity of the staining was controlled by digesting some sections with Streptomyces hyaluronidase in the presence of protease inhibitors before staining, or preincubating the bHABP probe with hyaluronan oligosaccharides.

The mean densities of immunopositive cells and vessels were quantified with 400-fold magnification from four random fields of each quadrant of the cardiac and kidney cross sections, and are given as a total for 1 mm². As the cross-sectional area of a kidney allograft may enlarge during tissue remodeling and formation of interstitial fibrosis, we aimed to estimate the absolute fibroproliferative activity by giving TGF-β1, anti-phospho-Smad2 and FSP-1 as immunopositive cell counts for total cross-sectional area. The area positive for HAS1, HAS2 and HA was measured with computer assisted imagining (Zeiss Axionvision 4.4, Carl Zeiss International, Oberkochen, Germany). Ten photographs with 400-fold magnification were taken from each sample and the average positive area of these ten measurements was used in statistical analyses. All analyses were performed in a blinded manner by two independent observers. For antibodies and dilutions used in the studies, please see Table 7.

**Fibrosis.** Cardiac fibrosis was determined in a blinded review by two observers from paraformaldehyde fixed paraffin sections stained with Masson’s trichrome and scored semiquantitatively (0 to 3) as follows: 0, no fibrosis; 1, mild fibrosis; 2, moderate fibrosis; and 3, severe myocardial fibrosis.
**Vasculopathy.** Cardiac allograft vasculopathy was determined from sections stained with hematoxylin-eosin and Resorcin-Fuchsin for internal elastic lamina using computer-assisted image processing (Zeiss Axiovision 4.4, Carl Zeiss International) and measuring the area between the internal elastic lamina and the vessel lumen. The percentage of arterial occlusion was determined as the ratio of neointimal area to internal elastic lamina area.

6. **mRNA expression**

**RNA isolation and reverse transcription.** Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription of mRNA was carried out from 100 ng total RNA using High-RNA-to-cDNA kit (Applied Biosystems Inc., Carlsbad, CA) in a total volume of 20 μl. After completed reverse transcription, 40 μl of PCR-grade water was added to each cDNA sample. Three μl of each sample (corresponding to 5 ng total RNA) were used in each subsequent PCR reaction.

**Quantitative real-time PCR.** Real-time PCR reactions were carried out on a RotorGene-6000 (Corbett Research, Hilden, Germany) using 2X DyNAmo Flash SYBR Green Master mix (Finnzymes, Espoo, Finland). The measurement of the PCR product was performed at the end of each extension period. Amplification specificity was checked using melting curve analysis. The number of mRNA copies of each gene of interest was calculated from a corresponding standard curve using the RotorGene software. The expression levels of mRNA were normalized against the housekeeping gene with the most stable expression as determined with GeNorm application (version 3.4). For primers used in the studies, please see Table 8.

7. **Microvascular dysfunction**

**Transmission electron microscopy.** For transmission electron microscopy, mid-axial myocardial and cortical samples were microdissected, fixed with 2.5% glutaraldehyde in 100 mM phosphate buffer, pH 7.4, incubated at +25 °C for 2 hours and post-fixed with 1% buffered osmium tetroxide for 1 hour, dehydrated and embedded in epon at room temperature. The sections were post-stained with uranyl acetate and lead and examined with a Jeol EX1200 II transmission electron microscope (Jeol Ltd., Tokyo, Japan) operating at 60kV. Cardiac interendothelial junctions were analyzed from 10-15 representative longitudinally cut microvascular vessels per each graft sample and the incidence of interendothelial gaps was quantified. Renal filtration barrier defects were quantified by the incidence of glomerular units comprising either detached endothelial cells or podocyte foot processes from glomerular basement membrane. Peritubular capillary gaps were quantified by the incidence of interendothelial tight junction loss. Images were acquired with an ES500W CCD camera (Gatan Corp., Pleasanton, CA).

**Microvascular interendothelial gap detection.** During heart removal, coronary arteries were perfused with 50 μl of rhodamine-labeled Concanavalin A lectin (red) (Vector Laboratories) that binds to the vascular endothelium and 50 μl of biotinylated *Ricinus communis* lectin (blue) (Vector Laboratories) that binds to the vascular basement membrane, both dissolved in 100 μl of NaCl. Thereafter the hearts were predisposed to 4-hour cold preservation, subsequent 1-hour warm ischemia and then the cardiac allograft coronary...
network was flushed with 200 μl 1% PFA in 0.05M citrate buffer, pH 3.5. Cryostat sections were stained with streptavidin and fluorescent-dye conjugate Avidin 350 nm (blue) (Promega Corp.) to detect biotinylated *Ricinus communis* lectin in order to identify inter-endothelial barrier disruption.

**Microvascular leakage.** A modified Miles Assay was used to measure extravasation of plasma proteins from the microvasculature into the interstitial space of heart and kidney. Immediately after reperfusion, the recipients were injected i.v. with Evans blue (Sigma-Aldrich) diluted with 0.9% NaCl, at a concentration of 30 mg/ml, which was allowed to circulate for 5 minutes (kidney) or 30 minutes (heart), whereafter the vascular network was flushed with 5 ml of 1% PFA in 0.05M citrate buffer, pH 3.5. For quantification of extravasated Evans blue, 100 mg of myocardial or cortical tissue was dissolved in 500 μl of formamide on a shaker at +60 °C for 24 hours. One hundred μl of formamide containing dissolved Evans blue dye was pipetted into cuvettes and the absorbance was measured by a spectrophotometer at 610 nm wavelength. To localize leakage, 1 minute before heart removal the coronary arteries were perfused with 50 μl of FITC-dextran 40 kD (*green*) dissolved in 100 μl of NaCl. At heart removal, the coronary network was flushed with 200 μl 1% PFA in 0.05M citrate buffer, pH 3.5. Extravasated FITC-dextran was localized from cryostat sections by its autofluorescence.

**Magnetic resonance imaging.** To assess tissue edema, we used magnetic resonance imaging (MRI) to analyze the transverse relaxation time (T2) value before heart transplantation or unilateral kidney clamping 4 hours after heart transplant reperfusion or 20 minutes after restoration of renal blood flow. The MRI studies were performed with a 4.7 T scanner (PharmaScan, Bruker BioSpin, Billerica, MA) using a 90 mm shielded gradient that is capable of producing maximum gradient amplitude of 300 mT/m with 80-μs rise time. A linear birdcage RF coil with an inner diameter of 60 mm was used. After a scout, T2-weighed fast spin echo (rapid acquisition with relaxation enhancement, RARE) sequence was used (TR/TEeff = 4200/55 ms, rare factor = 8, matrix size = 256 x 256, field-of-view = 30 x 30 mm, 15 slices, slice thickness = 2 mm). The multi- spin multi-echo sequence (MSME), based on CPMG (Carr-Purcell Meiboom-Gill) spin echoes was used for the determination of T2 [repetition time (TR) = 1500 ms, echo time (TE) = 11 - 220 ms and 20 echoes, number of averages = 2, matrix size = 256 x 192, field of-view = 70 x 70 mm, single slice with slice thickness = 2.0 mm, acquisition time = 9 min 36s]. The value of T2 was calculated by fitting the measured intensities to the exponential relaxation curve using the least-squares method.

**Microvascular perfusion.** Endothelium binding FITC-labeled *Lycopersicon esculentum* (Tomato) lectin (Vector Laboratories) was used to detect perfused vessels 5 minutes (kidney) or 30 minutes (heart) after re-establishment of circulation. Coronary arteries or the left renal artery were perfused with 50 μl FITC-labeled Tomato lectin diluted with 150 μl of 0.9% NaCl for 1 minute before removal of the hearts and kidneys for fluorescence microscopy analysis. The number of FITC+ microvascular vessels from mid-axial cryostat cross sections was analyzed by fluorescence microscopy by counting positive capillaries from four random fields of each quadrant of the cross section with 400-fold magnification, and is given as a total for 1 mm².
Laser Doppler monitoring. Tissue perfusion was analyzed by dynamic measurement with laser Doppler monitor, Transonic BLF21-Series (Transonic Systems Inc., Ithaca, NY). The moving Doppler effect was calculated via a 18 gauge (1.2 mm) probe head receiving fiber optic light reflected by stationary structures within the tissue as well as by moving particles (red blood cells). A low intensity beam of monochromatic light was emitted to the portion of myocardial or cortical tissue, to 1mm depth, and the tissue volume under laser Doppler monitoring was approximately 1 mm³. The flow signal was sampled 200 s⁻¹ from transmitted 19200 baud (=pulses per second) and is represented as tissue perfusion units (TPUs) that are relatively proportional to ml/min/100g of tissue. Tissue perfusion was analyzed before heart transplantation or unilateral kidney clamping and continuously for one hour after heart transplant reperfusion or continuously for 30 minutes, and at 6 hours and 3 days after restoration of renal blood flow.

The enzyme-linked immunosorbent assay (ELISA) of syndecan-1. For syndecan-1 measurements in serum samples, we used solid-phase ELISA kits (SEB966Ra, Design by Cloud-Clone Corp. Houston, TX, assembled by Uscn Life Science Inc., Cologne, Germany). The plates were analysed using the Bio-Rad microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA) at a wavelength of 450 nm. Frozen (-20°C) serum samples were thawed and shortly spun. 100μl of standard, blanco, and diluted samples (1:8 in 0.01M PBS) was used per well. Standard curves were constructed by plotting the concentration and mean absorbance at 450 nm. To calculate the concentration, the standards were fitted with a second-order polynomial equation.

8. Gene transfer

Production of recombinant AAV2 vectors and their in vitro expression analysis. The coding regions of the PDGF cDNAs were cloned into the psub-CMV-WPRE AAV2 expression vector. The rAAVs were produced with the two-plasmid transfection method as described. The titers of the recombinant AAVs were 5x10¹¹-10¹² genomic particles/ml. The expression of the recombinant proteins was verified by metabolically-labeled 293T cells transduced with the rAAVs. The expression of the proteins was analyzed by co-precipitation with the respective receptor-Ig molecules and the resultant protein complexes were separated in SDS-PAGE.

AAV2–mediated human PDGF-A-D gene transfer. After harvesting, coronary arteries of the donor rat heart were perfused through the aorta with 200 μl of recombinant human AAV2-PDGF-A, -B, -C or -D (1.0x10¹¹ gp) genes or AAV2 encoding EGFP (1.0x10¹¹ gp) as a control gene, and 50 μl of 10⁻⁷ mol/L acetylcholine to permeabilize the endothelium. AAV2-mediated human PDGF-A-D transgene expression in rat cardiac allografts was confirmed by a construct specific forward primer (5'-GGATGTTGCTTCTTTTACTTCTAGG-3') and backward primers specific for human PDGF-A (5'-AATGACCGTCCTGGTCTTGCAG-3'), PDGF-B (5'-CTCGGCCCCATCTTTTCTCC-3'), PDGF-D (5'-TGCTTGGGACACATTGACAT-3'), and PDGF-D (5'-ACAGCCACAATTTTCCCTC-3'). The relative intensity of AAV2-EGFP transgene expression was determined from allograft midline cross sections using computer-assisted image processing (Zeiss Axiovision 4.4, Carl Zeiss GmbH).

Adenovirus-mediated human PDGF-BB gene transfer. Replication-deficient E1-E3-
deleted human clinical grade (first generation, serotype 5) adenoviruses were produced under GMP conditions in 293T cells and analyzed to be free from contaminants (Makinen et al. 2002). After allograft procurement, coronary arteries of the donor rat heart were perfused through the aorta with 200 μl of adenoviruses encoding β-Galactosidase marker gene (LacZ) (1.0x10^9 pfu / ml) or recombinant human full-length (carrying retention motif) PDGF-BB (1.0x10^9 pfu / ml) under control of the cytomegalovirus (CMV) promoter. Transgene expression was confirmed by immunohistochemical staining of β-galactosidase marker gene and quantitative PCR of human PDGF-B mRNA expression in allografts.

9. In vitro assays

EC and SMC gene expression. Human cardiac microvascular endothelial cells (HMVEC-C) were cultured in EGM-2 MV growth medium. The cells used for the experiments were between passages 4-8. Rat coronary artery SMC (kindly provided by Dariusz Leszczynski; Finnish Centre for Radiation and Nuclear Safety, Helsinki, Finland) were cultured in 5% fetal calf serum. Simvastatin was dissolved in EtOH and activated by treatment with NaOH followed by neutralization to pH 7. HMVEC-C and rat coronary artery SMC were supplemented with activated simvastatin at a concentration of 1.0 μM for 72 hours. RNA isolation and real-time RT-PCR analysis were performed as described above.

TGF-β1-induced endothelial-to-mesenchymal transition in human cardiac microvascular endothelial cells. TGF-β1 was used to induce EndMT in HMVEC-C at a concentration of 10 ng/ml. Simvastatin was dissolved in EtOH and activated by treatment with NaOH followed by neutralization to pH 7 and concentrations of 0.1, 0.5 and 1.0 μM were used to inhibit EndMT. Antibodies used in in vitro studies were CD31 (ab9498, Abcam), ZO-1 (61-7300, Invitrogen, Carlsbad, CA) and Calponin (C2687, Sigma-Aldrich). HMVEC-C and EGM-2 MV growth medium were from Lonza, Basel, Switzerland. The cells used for the experiments were between passages 4-8. For immunofluorescence microscopy the cells were grown on glass coverslips for the indicated times. Coverslips were then washed three times with PBS, and the cells were fixed in ice-cold methanol at -20 °C. After washing three times with PBS, the cells were incubated in Dulbecco's PBS containing 3% BSA to prevent nonspecific binding of the antibodies. The cells were then incubated with the primary antibody in Dulbecco's PBS for 1 hour. The bound antibodies were detected using Alexa Fluor-594 secondary antibodies (Molecular Probes, Invitrogen). The coverslips were finally washed in water, mounted on glass slides using Vectashield anti-fading reagent (Vector Laboratories) and examined under an Axioplan 2 imaging microscope (Zeiss) using a 40x objective. Images were acquired with an AxioCamHRm camera and Axiovision 4.6 software (Zeiss) at the Molecular Imaging Unit of the University of Helsinki. For RNA isolation and real-time RT-PCR analysis, RNeasy mini kit (Qiagen) was used to isolate total cellular RNA. Reverse transcription was carried out with random hexamer primers (Invitrogen) and Superscript III reverse transcriptase (Invitrogen) using 1.0 μg of total RNA according to the Manufacturer's instructions. The cDNAs were amplified using TaqMan Assays-on-Demand gene expression products (Applied Biosystems) and GeneAmp 7500 Sequence Detector thermal cycler (Applied Biosystems). Control amplifications directly from RNA were performed in order to rule out DNA contamination. The levels of gene expression were determined using the Ct method and the results are shown as mRNA expression levels normalized to the levels of a gene with a constant expression (TBP, tata binding protein). SDS- PAGE and immunoblotting were performed after the cells were lysed in RIPA buffer.
Equal amounts of protein were separated by SDS-PAGE under reducing conditions using 4-20% gradient Tris-glycine gels (Lonza). The proteins were transferred to Protran nitrocellulose membranes (Whatman plc., Kent, United Kingdom) using a semi-dry blotting system (BioRad, Hercules, CA).

**Weibel-Palade body exosytosis in human blood endothelial cells.** Human dermal blood endothelial cells (BEC, PromoCell GmbH, Heidelberg, Germany) were cultured on fibronectin coated plates for 24-48 hours, changed to new growth media (PromoCell) and transferred to hypoxia (1% O<sub>2</sub>) or normoxia. Simvastatin was dissolved in EtOH and activated by treatment with NaOH followed by neutralization to pH 7 and a concentration of 1.0 μM was used to inhibit the secretion of Weibel-Palade body factors Ang-2 and ET-1. The growth media was collected at 24 hours. No apparent membrane disruption or cell death was observed by controlling cover slip cell counts at the end of the experiments and by using DAPI nucleic acid stain. ELISA (Quantikine-R&D Systems, Minneapolis, MN) was used to detect the presence of human angiopoietin-2 (DANG20) and human endothelin-1 (DET100) in BEC growth media. The growth media was diluted at 1:5.

**Allogenic mixed leukocyte culture and ELISpot.** Resident peritoneal macrophages were isolated from DA rats by injecting 10 ml sterile PBS (Ca<sup>2+</sup> and Mg<sup>2+</sup> free) and 10 ml air into the peritoneal cavity followed by gentle massage of the abdomen. Abdominal skin was cut and the resulting peritoneal fluid collected. The cells were centrifuged at 400 × g at +4°C for 10 minutes, washed once with PBS, dissolved in sterile water for 15 seconds to lyse any contaminating erythrocytes, and finally, the cell pellet was taken up in DMEM (10% fetal calf serum, 1% penicillin-streptomycin, and 1% glutamine). 1 × 10<sup>6</sup> macrophages were plated on 96-well cell culture plates in culture medium and allowed to adhere for 2 to 4 hours. Nonadherent cells were washed off with PBS, and new culture medium was added.

Splenocytes (T cells) were isolated from the spleen of WF rats with mechanical homogenization. The cell suspension was gravity-filtered through a 70-mm nylon mesh (BD FalconTM, San Diego, CA) to remove large debris. The cell suspension was centrifuged at 400 x g for 10 minutes in the cold, dissolved in sterile water for 15 seconds to lyse any contaminating erythrocytes, and finally, the cell pellet was taken up in culture medium. The cells were cultured at 2 × 10<sup>5</sup> cells per well on 96-well cell culture plates with previously isolated macrophages in DMEM. After 5 days of culture the wells were counted and diluted to concentrations of 60 000 cells / ml for the subsequent ELISpot analysis.

The cells were centrifuged at 400 x g for 5 minutes and resuspended in DMEM (10% fetal calf serum, 1% penicillin-streptomycin, and 1% glutamine). IFN-γ ELISpot (3220-2AW-Plus, Mabtech, Nacka Strand, Sweden) was performed according to the Manufacturer’s instructions. 1.0 x 10<sup>4</sup> cells (n=3/group) were plated per well and incubated for two hours at +37°C with 5% CO<sub>2</sub>. Anti-CD3 stimulation was used to assure the viability of the cells and native and syngenic cells were used as negative controls (n=3/group). The spots were counted with the ELISpot-reader (BIO-SYS-GmbH, Karben, Gemany).

**10. Statistics**
All data are given as mean ± SEM or as box plots showing the upper extreme (excluding outliers), upper quartile, median, lower quartile, and lower extreme (excluding outliers) where the outliers are shown as circles outside the box or as scatter plot showing mean and analyzed with SPSS 15.0 (SPSS Inc., Somers, NY). In non-parametric comparison, Mann-Whitney U test was used for two-group comparison and Kruskall-Wallis with Dunn test when multiple groups were compared to control. Dunn post hoc test was applied only if Kruskall-Wallis test demonstrated an overall statistically significant difference. In parametric comparison, Student’s t-test was used for two-group comparison and ANOVA with Dunnett’s correction for multiple group comparison. For comparison in a longitudinal study, data was analyzed by repeated-measures ANOVA. Linear regression analysis was applied to evaluate the relation of different simvastatin concentrations to the expression of mesenchymal genes in vitro. For survival Kaplan-Meier with Log-rank (Mantel-Cox) was applied. P<0.05 was considered as statistically significant.
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**rev** GCAATGATAAGGCTTCTCACCTGT

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**rev** AGAATCAGACGCTGCTGAGATTGAGG  
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PDGF-B  
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44
Normal = rat primers; Italic = human primers; fwd = forward sequence; rev = reverse sequence

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RESULTS

1. Transplant ischemia-reperfusion injury in cardiac allograft results in rapid microvascular dysfunction and fibroproliferation (unpublished results)

In transplantation, Tx-IRI leads to microvascular injury, which is a major cause for primary or late graft dysfunction. Similar mechanisms that induce microvascular dysfunction are also behind endothelial-to-mesenchymal transition (EndMT) and fibroproliferation in cardiac and kidney allografts. Recent findings suggest that relatively high numbers of fibroblasts in kidney and cardiac allografts arise through local EndMT (Zeisberg et al. 2007; Zeisberg et al. 2008). Interestingly, both microvascular permeability and EndMT, depend on endothelial cytoskeletal rearrangement, loss of endothelial-endothelial integrity and detachment from supporting basal lamina. Moreover, infiltration of peripheral blood mononuclear cell into allografts induces the expression of microvascular destabilizing and pro-inflammatory and pro-proliferative factors, e.g. HIF-1α and TGF-β1, which participate in endothelial barrier disruption and induce mesenchymal transition and fibrosis (Higgins et al. 2007; Li et al. 2009; Qi et al. 2011).

To investigate the effect of cardiac allograft Tx-IRI on microvascular dysfunction and fibroproliferation, intra-abdominal heterotopic heart transplantations were performed between fully MHC-mismatched rats. Cardiac allografts were subjected to 4-hour cold preservation, mimicking clinical organ transportation time. Warm ischemia occurring during heart transplantation was standardized to one hour that mimics warm ischemia during surgical procedure. Cardiac allografts were harvested five minutes after reperfusion to investigate microvascular permeability and perfusion disturbances. Further, recipients with 10-day follow-up were given CsA background immunosuppression to prevent severe episodes of acute rejection and to allow the development of alloimmune response.

Profound vascular leakage was present at the sites of interendothelial gaps of cardiac allografts already five minutes after reperfusion (Figure 6A). In addition, hypoperfusion and myocardial ischemia were observed (Figure 6B). At 10 days after reperfusion, pathological fibroproliferation that possibly involved EndMT was present in the border zone of fibrotic areas in cardiac allografts (Figure 6C-D).
Figure 6. (A) Immunofluorescence triple staining of cardiac allografts showed that the 4-h cold plus 1-h warm ischemia induces vascular leakage at the sites of endothelial barrier dysfunction revealed by leakage of FITC-dextran 40 kD (green) around the endothelial-endothelial cell gaps (*Risin Communis lectin*; blue) in perfused microvascular vessels (*Rhodamine-labeled Concavalin A lectin*; red) already five minutes after reperfusion. (B1) Hypoperfused areas co-localized with (B2) pimonidazole-positive ischemic myocardium five minutes after reperfusion. (C-D) We also noticed that p-Smad-2 immunoreactivity, indicating active TGF-β1 signaling and fibroblast specific protein (FSP)-1 and endothelial marker RECA-1 double-immunoreactivity, that may contribute to allograft fibrosis through EndMT, localized in cardiac allograft perifibrotic boarderline at 10 days. Arrowhead = fibrosis, arrow = perifibrotic boarderline, double arrow head = myocardial with obvious microvascular network. Unpublished data.
2. Donor simvastatin treatment targets microvasculature of the heart, kidney, and liver in the rat (I, II)

Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are widely used for primary and secondary prevention of cardiovascular diseases. In addition to their lipid lowering ability, statins have beneficial pleiotropic microvasculoprotective effects. These drugs seem to have anti-inflammatory, antioxidative, and -thrombotic effects (Koh 2000; Takemoto and Liao 2001). Statins may also attenuate the expression and secretion of angiogenic growth factors and microvascular activation at the site of injury (Undas et al. 2006). To date, however, the role of statins in Tx-IRI in cardiac and kidney allografts remain poorly understood.

In our study, we focused on the effects of donor simvastatin treatment on microvascular function. The rapid protective effects of statins could have novel clinical implications in organ transplantation where the time-window to treat a cadaveric organ donor is limited – usually between 6 to 8 hours from the declaration of brain-death to organ procurement. After peroral administration, simvastatin is absorbed in the intestine, and the inactive lactone form is metabolized to the active β-hydroxy acid form in the liver before proper HMG-CoA reductase inhibition is achieved in the target tissue.

To establish the clinical relevance of oral donor simvastatin treatment in the terms of adequate absorption and metabolism, we performed detailed pharmacokinetic analyses of the blood levels of the inactive and active simvastatin forms after (i) simvastatin 5 mg p.o. administration to rat donors and (ii) simvastatin 80 mg administration via nasogastric tube to brain-dead human organ donors. In both situations, simvastatin was absorbed and metabolized to the active β-hydroxy acid form within 2 hours, but the metabolism was more rapid in the rat than in man (Figure 7A-B). In the rat, the simvastatin doses were based on our dose response study. Immunohistochemical and immunofluorescense double-stainings revealed that the simvastatin target molecule HMG-CoA reductase was mainly found in the microvasculature of the heart (Figure 7C), kidney and liver in the rat (Tuuminen et al. 2013).
Figure 7. (A) Pharmacokinetic analysis with liquid chromatography-ionspray tandem mass spectrometry showed that after rat single-dose peroral simvastatin 5.0 mg/kg administration, simvastatin lactone was absorbed rapidly within hours. (B) Simvastatin lactone was also absorbed within 2 hours in human organ donors after clinically-approved simvastatin 80 mg administration via nasogastric tube indicating the applicability of donor simvastatin treatment in a clinical situation. (A-B) Simvastatin lactone was hydroxylated to its active β-hydroxy acid form and was eliminated much more rapidly in the rat than in human organ donors. (C) HMG-CoA reductase, a target molecule of statins, localized in donor hearts abundantly in the cardiac capillary network of non-transplanted rat hearts, but not in vSMC nor in cardiomyocytes, suggesting capillary network as the primary therapeutic target. (A-C) reprinted from Tuuminen R et al., Circulation 2011 Sep 6;124(10):1138-50 with the permission from the American Heart Association and Lippincott Williams and Wilkins (LWW).

3. Donor simvastatin treatment prevents microvascular activation and instability during cold and warm ischemic preservation of hearts and kidneys in the rat (I, II)

Cytoskeletal rearrangement of microvascular endothelial cells and their surrounding pericytes may lead to increased microvascular permeability, leukocyte extravasation, thrombosis, vasoconstriction, and compromised tissue perfusion. This may be mediated through stress-fiber formation and also through the release of vasoactive factors such as angiopoietin (Ang)-2 and endothelin (ET)-1 from endothelial Weibel-Palade bodies. Statins, on the other hand, have important pleiotropic vasculoprotective effects mediated largely through the inhibition of important regulators of cell cytoskeleton and intracellular signaling pathways. Therefore, we studied phosphorylation of intracellular myosin light chain (MLC)2 in the vascular wall, Rho GTPase signaling and Ezrin/radixin/moesin (ERM) family of actin-binding proteins that function as signal transducers in responses involving cytoskeletal rearrangement.

Four-hour (heart) or 16-hour (kidney) cold preservation and additional 1-hour warm ischemia resulted in abundant vascular RhoA/ROCK activation indicated by immunoreactive phosphorylated adducin in capillaries, postcapillary venules, arterioles and arteries in the heart (Figure 8A-8C) and kidney (Figure 9B). Donor simvastatin treatment given 2 hours before the procurement prevented vascular RhoA/ROCK activation in the heart (Figure 8A-8C) and kidney (Figure 9B). Furthermore, donor simvastatin-treated prevented vascular wall activity of other cytoskeletal rearrangement and stress fiber formation involved molecules MLC2 (Figure 9A) and ERM proteins in allografts.

Next, we studied the effects of donor simvastatin treatment on structural and molecular changes in the microvasculature. Ultrastructural analysis with transmission electron microscopy (TEM) revealed that 4-hour (heart) or 16-hour (kidney) cold preservation and additional 1-hour warm ischemia resulted in myocardial and peritubular capillary interendothelial gap formation, and glomerular endothelial and pericyte detachment from supporting glomerular basement membrane (Figure 8D and 9C). Donor simvastatin treatment, however, preserved myocardial capillary and peritubular and glomerular microvascular barrier integrity during cold and warm ischemia (Figure 8D and 9C) and prevented vascular permeability and perfusion disturbances that occur early after kidney allograft reperfusion.
Figure 8. (A-C) To determine whether cardiac 4-h cold and 1-h warm ischemia lead to RhoA/ROCK activation, we performed immunostainings for phosphorylated adducin (p-adducin) in native DA hearts or hearts subjected to 4-hour cold and 1-hour warm ischemia. Four-hour cold and 1-hour warm ischemia induced p-adducin immunoreactivity in RECA-1⁺ ECs, NG2⁺ pericytes and α-SMA⁺ smooth muscle cells of capillaries, postcapillary venules, arterioles, and arteries. (D) Microvascular EC-EC gaps (arrows) were determined by transmission electron microscopy of left ventricular longitudinally cut microvascular vessels. Transmission electron microscope analysis indicated that 4-hour cold and 1-hour warm ischemia markedly increased the incidence of microvascular EC-EC gaps. (A-D) Donor simvastatin treatment decreased p-adducin immunoreactivity in capillaries, postcapillary venules, and arterioles, as well as in the media of arteries, and decreased the formation of EC-EC gaps, indicating decreased RhoA/ROCK activation and enhanced endothelial stability. (A-D) reprinted from Tuuminen R et al., Circulation 2011 Sep 6;124(10):1138-50 with the permission from the American Heart Association and Lippincott Williams and Wilkins (LWW).
Figure 9. (A-B) To determine whether kidney 16-h cold and 1-h warm ischemia lead to cell cytoskeleton signaling pathways, we performed immunostainings for phosphorylated myosin light chain (p-MLC)2 and phosphorylated adducin (p-adducin) in native DA kidneys or kidneys subjected to 16-hour cold and 1-hour warm ischemia. Sixteen-hour cold and 1-hour warm ischemia induced microvascular p-MLC2 and p-adducin immunoreactivity, both of which were decreased by donor simvastatin treatment. (C) Glomerular filtration barrier defects were determined by transmission electron microscopy. Transmission electron microscope analysis indicated that 16-hour cold and 1-hour warm ischemia markedly increased the incidence of detachment of fenestrated endothelium and podocytes from glomerular basement membrane. Simvastatin maintained glomerular ultrastructure during cold and warm ischemia, and the fenestrae of the endothelium did not reach over 100 nm in most of the glomerular units. (A-C) reprinted from Tuuminen R et al., Am J Transplant. 2013 Aug;13(8):2019-34 with the permission from the American Heart Association and Lippincott Williams and Wilkins (LWW).

4. Donor simvastatin treatment inhibits permeability and perfusion disturbances in IRI in a kidney artery clamping model (unpublished results)

As simvastatin reduced RhoA/ROCK signaling and preserved barrier integrity of microvascular network during prolonged cold ischemic preservation and warm ischemia, we
proceeded to study the effects of simvastatin pretreatment on IRI in non-allogenic and allogenic environment. First, we used a kidney artery clamping as a model for renal IRI. Single-dose peroral simvastatin treatment (5 mg/kg) 2 hours before renal 30-minute warm ischemia, maintained outer medullar T2-weighted relaxation time in a clamped ischemic right kidney and also non-clamped contralateral left kidney 20 minutes after reperfusion (Figure 10A). Histological evaluation of the kidney at the 20-minute timepoint revealed intense intravascular congestion, capillaries filled with trapped erythrocytes in vehicle-treated control rats, but not in simvastatin-treated rats (Figure 10B). Further analysis using albumin-binding Evans blue dye revealed that simvastatin treatment reduced vascular leakage 5 minutes after reperfusion in a dose-dependent manner (Figure 11A). Endothelium-binding FITC-labeled tomato lectin perfusion confirmed poor post-ischemic blood flow 5 minutes after reperfusion in vehicle-treated kidneys (Figure 11B). Simvastatin treatment dose-dependently improved peritubular capillary blood flow (Figure 11B). The effect of simvastatin on peritubular capillary blood flow was counteracted by RhoA supplementation with GGPP (Figure 11B), indicating that simvastatin regulates microvascular blood flow via inhibition of RhoA signaling pathway.

**Figure 10.** DA rats were treated with simvastatin 2 hours before 30-minute right renal artery clamping. (A) 20 minutes after reperfusion, non-invasive magnetic resonance imaging (MRI) analysis and (B) histological evaluation of H&E stained sections were performed to study signal intensities related to diffusion of water and fluids of T2-weighted images and their relationship to specific histopathologic tissue characteristics. Unpublished data.
5. Donor simvastatin treatment preserves tubular tight junctions and reduces tubular adhesivity in renal allografts during ischemia-reperfusion injury (unpublished results)

Next we studied whether donor simvastatin treatment with its direct vasculoprotective effects could have beneficial effects also at tubular level. Donor simvastatin treatment preserved expression of tubular junctional proteins ZO-1 and Occludin in kidney allografts and abrogated adhesion molecule P-selectin induction, when compared to non-treated kidney allografts (Figure 12).
Figure 12. Kidney allograft donors were treated with single-dose peroral simvastatin (5 mg/kg) or control vehicle. Allografts were subjected to 16-h cold preservation and 1-h warm ischemia and transplanted to MHC-mismatched recipients. Six hours after allograft reperfusion, tubular junction (ZO-1 and Occludin) and adhesion (P-selectin) proteins were evaluated by immunohistochemistry. Unpublished data.

6. Donor simvastatin treatment reduces myocardial and tubulointerstitial injury in cardiac and renal allografts during transplant ischemia-reperfusion injury (I, II)

Statin medication is conventionally commenced after heart and kidney transplantation due to its cardiovascular protection and beneficial effects on chronic rejection and long-term survival (the Scandinavian Simvastatin Survival Study 1994; Kobashigawa et al. 1995; Shepherd et al. 1995; Heart Protection Study Collaborative 2002). However, previous observations from randomized clinical trials support the short-term effects of preoperative statin administration against IRI-induced cardiac and kidney cell damage in a cholesterol-independent fashion when administered only hours or few days before elective intervention.

To evaluate Tx-IRI-induced myocardial and tubulointerstitial damage in cardiac and kidney allografts, clinically used biomarkers such as cardiac troponin T (cTnT) and neutrophil gelatinase-associated lipocalin (NGAL) were applied. In the cardiac transplantation model, we treated the donor (5 mg/kg), the recipient (2 mg/kg), or both the donor and the recipient with simvastatin. Six hours after reperfusion, serum was collected for cardiac troponin T measurement. Prolonged 4-hour cold ischemia increased serum release of cardiac troponin T in allograft recipients, which was nearly prevented by donor, but not by recipient simvastatin treatment (Figure 13A). In the recipients of kidney allografts, 16-hour cold ischemia induced serum NGAL levels both in simvastatin and vehicle-treated control allograft at 6 hours (Figure 13B). However, the NGAL levels returned towards normal in the recipients with allografts from donors treated with simvastatin already at 5 days, indicating faster recovery from tubulointerstitial injury (Figure 13B).
Figure 13. Cardiac allograft donors (5 mg/kg), recipients (2 mg/kg), or both donors and recipients, and kidney allograft donors (5 mg/kg) were treated with single-dose peroral simvastatin or control vehicle. (A-B) serum cardiac troponin T (cTNT) levels were analyzed 6 hours and neutrophil gelatinase-associated lipocalin (NGAL) levels 6 hours and 5 days after reperfusion to evaluate myocardial damage after heart transplantation and tubulointerstitial damage after kidney transplantation, respectively. (B) reprinted from Tuuminen R et al., Am J Transplant. 2013 Aug;13(8):2019-34 with the permission from the John Wiley & Sons, Inc.

7. Donor simvastatin treatment targets the expression of multiple genes involved in vascular homeostasis, fibroproliferation, and innate and adaptive immune responses in cardiac and renal allografts in the rat (I-III)

Rat cardiac and renal allografts are predisposed to nonimmunological and immunological injuries during donor brain-death, cold ischemic preservation and warm ischemia, ischemia-reperfusion injury, and during the activation of innate and adaptive immune responses after transplantation. These may alter the expression of a variety of genes involved in vascular homeostasis, cell survival, inflammatory and fibroproliferative responses including arteriosclerosis, interstitial fibrosis, glomerular sclerosis, and tubular atrophy in allografts. Simvastatin treatment may have direct and indirect effects on the expression of these genes. Thus, we analyzed the effect of simvastatin treatment on the mRNA expression levels using quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) with in vivo and in vitro models. In cardiac allografts subjected to 4-hour cold and 1-hour warm ischemia, donor simvastatin treatment decreased the mRNA levels of Rho GTPases RhoA and Rac1 and had an influence on genes involved in vascular homeostasis, such as HIF-1α and its downstream iNOS, VEGF, HO-1, and ET-1. In addition to quantitative real-time RT-PCR, donor simvastatin treatment significantly decreased nuclear HIF-1 immunoactivity in cardiac allografts. In vitro analysis revealed that simvastatin treatment modulates mRNA levels of genes involved in vascular homeostasis through endothelial but not smooth muscle cells.

In kidney allografts with 16-hour cold and 1-hour warm ischemia, donor simvastatin treatment reduced the mRNA expression of Ang-2, a potent mediator of vascular destabilization and its receptor Tie-2, arginase-1, the enzyme that metabolizes a NO synthase substrate arginine, the pro-apoptotic BAX/Bcl-2 ratio, pro-fibrotic and vasoactive factors such as ET-1, PDGF-A and TGF-β1, tissue remodeling MMP-9, innate immune ligand HAS-1, lymphangiogenic VEGF-C and VEGFR-3, and dendritic cell maturation markers CD80,
CD83 and CD86, and MHC class II 6 hours after reperfusion (Table 8), when compared to allografts from vehicle-treated donors. At 5 days, donor simvastatin treatment significantly downregulated the mRNA levels of transcription factor HIF-1α, its downstream iNOS and VEGF as well as innate immune response receptor TLR-2 and its downstream NF-κB (Table 9).

Table 9. Quantitative real-time RT-PCR analysis of mRNA expression of genes involved in vascular homeostasis, fibroproliferation, and innate and adaptive immune responses in renal allografts in the rat

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Donors were treated with simvastatin 5 mg/kg p.o. 2 hours before graft harvest. Kidney allografts were collected 6 hours or 5 days after reperfusion for quantitative real-time RT-PCR. The results were normalized to 18S rRNA and are given as a ratio to mRNA in control allografts at 6 hours. Modified from Tuuminen R et al., Am J Transplant. 2013 Aug;13(8):2019-34 with the permission from the John Wiley & Sons, Inc.

The beneficial effects of donor simvastatin treatment on microvascular permeability in cardiac allografts and perfusion in cardiac and kidney allografts were mediated through nitric oxide (NO) and inhibition of the RhoA GTPase pathway, respectively ((Tuuminen et al. 2011) and Figure 11B).

8. Donor simvastatin treatment inhibits chronic rejection and late graft loss of cardiac allografts in the rat (I, unpublished results)

The gradual development of allograft vasculopathy is one of the main reasons for the long-term allograft failure. Allograft vasculopathy is characterized by concentric neointimal formation in the vasculature of the transplant. Among vasculopathy, chronic inflammation and fibroproliferation eventually lead to cardiac fibrosis and graft failure. Statin administration to the recipients delays the progression of allograft vasculopathy and reduces
the rate of graft failure both through its cholesterol-dependend and cholesterol-independent anti-inflammatory and anti-mitogenic effects (Kobashigawa et al. 1995; Wenke et al. 1997; Wenke et al. 2003; Kobashigawa et al. 2005). Here we studied the effect of single-dose donor, daily recipient and combined donor and recipient simvastatin treatment on cardiac allograft inflammation, fibrosis, micro- and macrovascular alterations and graft survival. The allograft recipients were treated with suboptimal dose of CsA to avoid irreversible episodes of acute rejections but to allow the chronic low-level inflammation in allografts and the development of chronic rejection. Allografts were followed until the study end-point 56 days after transplantation. Donor or combined donor and recipient simvastatin treatment significantly reduced the densities of allograft infiltrating ED-1+ macrophage, MPO+ neutrophil and CD4+ T cell, and myocardial fibrosis. However, only combined donor and recipient simvastatin treatment significantly improved allograft survival, maintained the density of RECA-microvasculature in the myocardium, and reduced allograft vasculopathy 56 days after the transplantation. The effect of simvastatin combination treatment on arterial occlusion was evident both in micro- (arterial occlusion percentage control vs. simvastatin combination treatment, diameter <50mm; 59.9% vs. 28.2%) and macrovessels (diameter >50mm; 54.3% vs. 28.0%, respectively, Figure 14).

Figure 14. Cardiac allograft donors were treated with single-dose simvastatin (5 mg/kg, p.o.) 2 hours before graft removal, recipients with single-dose simvastatin (2 mg/kg, p.o.) 2 hours before graft reperfusion and daily thereafter (2 mg/kg/d, p.o.), or simvastatin was given to both donors and recipients. CsA background immunosuppression was given to the cardiac allograft recipients as 2.0 mg/kg for 7 days and 1.0 mg/kg daily thereafter. To determine arterial occlusion of micro- and macrovessels in chronically-rejecting cardiac allografts we performed morphometric analysis 56 days after transplantation. Arrows indicate neointimal area. Scale bars=50 μm.. Partially unpublished data.

9. Persistent overexpression of PDGF-A, -C and -D induces cardiac allograft vasculopathy and fibrosis, whereas in ischemia-reperfusion injury PDGF-B prevents microvascular loss and PDGF receptor inhibition induces myocardial injury in rat cardiac allografts (IV, unpublished results)

Rat cardiac allografts subjected to chronic rejection in MHC-mismatched recipients were analyzed 56 days after transplantation. Chronic rejection induced intense PDGF ligands and receptors expression in the allograft coronary artery intima, with the exception of PDGF-B. To further elucidate the role of different PDGF ligands in cardiac allograft chronic rejection, we chose adeno-assosiated virus (AAV)-2 gene transfer and 100 day transplantation model,
to ensure stable and long-term transgene expression. AAV2-mediated gene transfer of PDGF-A, -C, and -D, but not -B, orchestrated TGF-β1 mRNA upregulation (Fraser et al. 2003), and pathological fibroproliferative manifestations in the allografts.

Next, to focus on the role of PDGF-B in cardiac allograft ischemia-reperfusion injury, coronary arteries of the donor hearts were perfused of adenoviruses encoding β-Galactosidase marker gene (LacZ) or recombinant human full-length PDGF-BB and the allografts were subjected to 2 hours cold ischemia before transplanted to MHC-mismatched recipients. At 10 days, ELISA analysis of serum syndecan-1, an indicator of endothelial glycocalyx shedding, was used to evaluate endothelial injury of cardiac allografts. Serum syndecan-1 levels were 20-fold higher in Ad.LacZ -perfused allografts when compared to serum levels of native rats (Figure 15A). In Ad.hPDGF-BB-perfused allografts serum syndecan-1 levels were significantly lower when compared to Ad.LacZ controls (Figure 15A). Quantitative real time RT-PCR revealed that hPDGF-BB gene transfer induced mRNA expression of BMP-7, an inhibitor of the endothelial-mesenchymal transition (Figure 15B) (Zeisberg et al. 2007), and anti-apoptotic Bcl-2 (Figure 15B). Next, we analyzed cardiac allograft capillary and artery densities. In control cardiac allografts RECA1+ myocardial capillary density was decreased when compared to native DA rat hearts. Gene transfer of hPDGF-BB preserved cardiac allograft RECA1+ endothelial phenotype (Figure 15C). The α-SMA+ vessel densities were not affected at 10 day by Ad.hPDGF-BB gene transfer (Figure 15D). PDGF receptor tyrosine kinase inhibitor imatinib mesylate increased endothelial glycocalyx shedding and cardiomyocyte injury evidenced by elevated serum syndecan-1 and cTnT (Figures 15E and 15F). Finally, in major MHC-mismatched mouse allograft donors treated with specific PDGFR-α and -β mAbs, inhibition of PDGFR-α, but not -β induced myocardial damage 6 hours after reperfusion, when compared to IgG controls (Figure 15 G).
Figure 15. First, cardiac allografts were perfused with adenoviral (Ad.) vectors containing either LacZ control gene or human PDGF-BB ligand. Cyclosporine A background immunosuppression was administered to the cardiac allograft recipients as 2.0 mg/kg for 7 days and 1.0 mg/kg daily thereafter. (A) Serum syndecan-1 levels, an indicator of endothelial injury, (B) mRNA expression levels of central genes involved in microvascular homeostasis, mesenchymal transition and survival and myocardial densities of (C) RECA-1⁺ capillaries and (D) α-SMA⁺ arteries were determined 10 days after transplantation. The inhibition of PDGF receptor activity in rat cardiac allograft ischemia-reperfusion on (E) endothelial and (F) cardiomyocyte injury was evaluated at 6 hours. (G) The effect of specific PDGFR-α and -β inhibition on mouse cardiac allograft myocardial injury after ischemia-reperfusion at 6 hours. Arrows indicate TUNEL⁺ apoptotic nuclei. Scale bars=50 μm. Unpublished data.
DISCUSSION

Microvasculoprotective effects of simvastatin

During static cold preservation, a transplant is predisposed to ischemia and loss of nutrients. Moreover, withdrawal from blood flow excludes a transplant from physiological pulsatile vascular wall shear stress. Both ischemia and metabolic changes together with loss of shear stress disturb vascular and parenchymal homeostasis (Figures 16 and 17). Microvascular dysfunction plays a critical role in Tx-IRI, allograft dysfunction and the development of untreatable allograft vasculopathy (Hollenberg et al. 2001; Marti et al. 2001; Tona et al. 2006; Kubrich et al. 2008). As the potential cellular effects of donor simvastatin treatment are carried along with the transplant, the possible primary targets are transplant tissue cells and passenger leukocytes. The exact role of different cell types in mediating the protective effects of statins in Tx-IRI in kidney and cardiac allografts is difficult to determine in vivo but several of our findings point out that pleiotropic cholesterol-independent microvascular protection has a pivotal role. First, the cholesterol levels of rats were low with a low-cholesterol diet, and the levels were not affected by simvastatin treatment. Second, immunohistochemical and immunofluorescence double-stainings revealed that the simvastatin target molecule HMG-CoA reductase was mainly expressed in the microvasculature of the rat heart and kidney. Third, donor simvastatin treatment upregulated the mRNA expression of multiple vasculoprotective genes during heart and kidney preservation and maintained microvascular integrity. Fourth, donor simvastatin treatment prevented the microvascular permeability and perfusion disturbances very early after reperfusion. Fifth, simvastatin inhibited the release of vasoactive peptides ET-1 and Ang-2 into the media of human blood endothelial cells in vitro. Sixth, donor simvastatin treatment together either with geranylglycerol pyrophosphate or with L-NAME demonstrated that the RhoA/ROCK and NO pathways are directly involved in the vasculoprotective effects of simvastatin in our models.
**Static cold preservation of transplant regulates multiple signaling pathways**

<table>
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<th>Ischemia</th>
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<td>[Diagram showing multiple signaling pathways]</td>
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**Figure 16.** Multiple signaling pathways regulating vascular homeostasis, inflammation, fibrosis and survival are affected by ischemia and loss of shear stress during allograft cold preservation. Downward arrowhead = downregulation of pathway activity by allograft cold preservation. Underline indicates factors affected by donor simvastatin treatment.

**Anti-inflammatory effects of simvastatin and methylprednisolone are divergent**

Until the initiation of immunosuppressive treatment targeting to the inhibition of T cell activation by calcineurin inhibitors, acute cellular rejections limited the use of cadaveric organ transplantations as an established treatment option for end-stage heart and kidney disease. However, malignancies, infections and cardiovascular side-effects of immunosuppressive medication are among major causes for mortality after transplantation. Statins mediate anti-inflammatory effects through HMG-CoA reductase and integrin leukocyte function-associated antigen (LFA)-1 -dependent pathways (Weitz-Schmidt et al. 2001). As donor simvastatin treatment may affect allograft passenger leukocytes, we investigated the effect of simvastatin on the phenotype and T cell stimulation properties of macrophages. Simvastatin treatment downregulated both TNF-α and iNOS (M1 macrophage factors) and Arg-1 (M2 macrophage factor), and did not alter T cell stimulation. These results suggest that donor simvastatin treatment may not alter the M1/M2 polarization of passenger leukocytes or their antigen presentation properties. MHC II mRNA expression of allografts, on the other hand, was downregulated already 6 hours after reperfusion, indicating decreased immunogenic potential. Overall, it seems that donor simvastatin treatment inhibited microvascular dysfunction, allograft damage, and activation of innate immunity and maturation of APC. We think this in turn decreases the priming of alloreactive T cells. Combined donor simvastatin and methylprednisolone treatment, however, resulted in microvasculoprotective and immunomodulatory effects (Figure 16). Methylprednisolone
reduced chemotactic and pro-inflammatory NF-κB, IL-8, MCP-1 and MIP-1β mRNA expression and combined with simvastatin, the treatment was more effective in reducing innate immunity cells neutrophil infiltration, myocardial injury and acute rejection than either treatment alone.

**Figure 17.** Ischemia-reperfusion injury (IRI) induces infiltration of inflammatory cell by microvascular cytoskeletal reorganization and expression of pro-inflammatory and innate immune ligands. Combined donor simvastatin and methylprednisolone treatment resulted in dual vasculoprotective and immunomodulatory effects. Simvastatin (italic) reduced the RhoA and Rac1 mRNA expression of RhoGTPases and decreased microvascular RhoA signaling, while methylprednisolone (bold) decreased myocardial danger ligand hyaluronic acid (HA) accumulation and expression of chemotactic cytokines IL-8, MCP-1, MIP-1β. The donor combination treatment efficiently reduced the number of allograft-infiltrating macrophages and neutrophils. Figure drawn by Simo Syrjälä.

**Antifibroproliferative effects of simvastatin**

Transforming growth factor (TGF)-β1 secretion is induced by many cell types during Tx-ischemia-reperfusion and rejection responses in cardiac and kidney allografts (Waltenberger et al. 1993; Zhao et al. 1994; Lario et al. 2003). TGF-β1 has been postulated to exert a broad spectrum of effects. TGF-β1 may protect the myocardium from IRI and mediate immunosuppressive effects (Dandapat et al. 2008). On the other hand, TGF-β1 may promote apoptosis, permeability and epithelial-to-mesenchymal (EMT) and endothelial-to-mesenchymal transition (EndMT) in hearts and kidneys (Behzadian et al. 2001; Bhowmick et al. 2001; Zeisberg et al. 2007). Donor simvastatin treatment decreased fibroproliferative activity and development of fibrosis in cardiac and kidney allografts. This was likely mediated both through direct and indirect effects. Direct antifibrotic effects may include inhibition of RhoA, which is necessary for TGF-β1 induced mesenchymal transition (Bhowmick et al. 2001); (Patel et al. 2006) (Figure 18). We found that simvastatin in a dose-dependent manner inhibited TGF-β1 induced loss of tight junctions and fibrotic phenotype in human cardiac microvascular endothelial cell cultures. Thus, we hypothesized that targeting endothelial stability may have antifibroproliferative potential. Also, donor simvastatin treatment reduced the expression of many pro-fibrotic genes, presumably mediated partly via
reduced endothelial activity and allograft infiltration of inflammatory cells, sources for bFGF, ET-1, PDGF-A and TGF-β1.

**Figure 18.** Endothelial–to-mesenchymal transition (EndMT) is a physiological process during heart and kidney development, but is not observed in quiescent adult vasculature. In transplantation, reorganization of the actin cytoskeleton and extracellular matrix by hypoxia-induced RhoA and inflammatory cytokine TGF-β1 play a pivotal role in endothelial cell plasticity to undergo EndMT. EndMT has a pathological role in cardiac and kidney allograft fibrosis. Donor simvastatin treatment inhibited both RhoA and TGF-β1 mRNA expression and down-stream signaling, and preserved myocardial capillary intensity, a possible antifibroproliferative mechanism through EndMT inhibition. Figure modified from Potenta S et al., British Journal of Cancer, 2008.

The role of PDGF ligands in cardiac allografts

In cardiac transplantation, angiogenic growth factors are triggered rapidly after a mechanical vascular injury or after a drop in oxygen level. If we postulate angiogenic growth factors to be protective during cardiac transplant I/R-injury, as was shown with myocardial ischemia-reperfusion and infarction models (Hsieh et al. 2006a; Hsieh et al. 2006b), ensuring endothelial viability or even pharmacological supplementation of endothelial derived angiogenic growth factors could facilitate protection from at least vascular SMC and cardiomyocyte death. Therefore, it is fascinating to speculate that angiogenic growth factors may have a bidirectional role in heart transplantation. On short term, they may mediate physiological protection of the heart against transplant I/R-injury, on the other hand; during prolonged upregulation they may direct pathological consequences, such as intimal formation via vSMC “neoplasia”. Here we showed that persistent overexpression of PDGF ligands -A, -C and -D induced TGF-β1 mRNA expression and chronic rejection in rat cardiac allografts. Yet, the role of PDGF ligands in ischemia-reperfusion of cardiac allografts remains unsolved.

Study limitations

Our cardiac and kidney transplantation models in rats and mice were performed exclusively between major MHC-mismatched recipients. Further, we used clinically relevant organ preservation and warm ischemia times, donor treatment was designed to fit the clinical use and background immunosuppression took care. However, our experimental models did not and cannot take into account all the variables involved in the clinical situation. The principal concern regarding our models is the lack of donor brain death. As donor treatment with
another lipophilic HMG-CoA reductase inhibitor, atorvastatin, for 2 days failed to protect kidney allografts from rejection in an extreme non-immunosuppression brain death model (Hoeger et al. 2012), it is justified to question whether the microvasculoprotective effects of donor simvastatin treatment or adenoviral PDGF-BB transgene expression withstand in even more severe models.

**Clinical implications**

Based on our studies, pharmacokinetics of donor simvastatin treatment fits to the narrow time-window to treat a cadaveric organ donor from the declaration of brain death to organ procurement. In contrast to recipient statin treatment, donor statin treatment targets the transplanted organ and the very proximal events that may lead to early allograft injury and predispose the organ to the development of delayed graft function and acute and chronic graft failure. Therefore, the vasculoprotective effects of statins may not be restricted to specific organs – important information when considering donor simvastatin treatment and multi-organ donation. We have therefore initiated a randomized clinical trial to investigate the effect of donor simvastatin treatment as an adjunct therapy on short- and long-term results of cardiac, lung, and kidney allografts.
CONCLUSIONS

Overall, we believe that the effect of donor simvastatin treatment is primarily due to cytoskeletal stabilization of microvascular endothelial cells and pericytes, which leads to decreased permeability and perfusion defects, infiltration of innate immune cells and IRI in the allografts. As a consequence of less tissue injury, the damage associated molecular patterns do not activate Toll-like receptors on passenger leukocytes and graft infiltrating macrophages, which leads to decreased antigen presentation on alloreactive T cells in secondary lymphoid organs and finally decreased infiltration of alloreactive T cells into the allografts. In multi-organ donor situation, simvastatin may offer a feasible microvascular-targeted adjunct therapy for organ protection by preventing activation of immune responses after Tx-IRI.
Mikrovaskulaari dysfunktio sydän- ja munuaissiirteen iskemia-reperfuusiovauriossassa

Sydän- ja munuaissiirteiden lyhyttä- ja pitkääikaista eloonjäämistä on viime vuosikymmeninä huomattavasti parannettu ja sen vuoksi potilaita listataan sydän- ja munuaissiirtoihin yhä enenevissä määrissä. Aikaisemmin elinluovuttajat olivat nuoria ja terveitä, mutta nykyisin aivokuolema on usein jopa aikuisia. Koska elinluovuttajien puute on johtanut tahaamattomuutta aivokuolemaan johtanut tapahtumaa, Koska elinluovuttajien puute on johtanut tahaamattomuutta aivokuolemaan johtanut tapahtumaa, koska elinluovuttajien puute on johtanut tahaamattomuutta aivokuolemaan johtanut tapahtumaa, koska elinluovuttajien puute on johtanut tahaamattomuutta aivokuolemaan johtanut tapahtumaa, koska elinluovuttajien puute on johtanut tahaamattomuutta aivokuolemaan johtanut tapahtumaa, koska elinluovuttajien puute on johtanut tahaamattomuutta aivokuolemaan johtanut tapahtumaa, koska elinluovuttajien puute on johtanut tahaamattomuutta aivokuolemaan johtanut tapahtumaa, koska elinluovuttajien puute on johtanut tahaamattomuutta aivokuolemaan johtanut tapahtumaa, koska elinluovuttajien puute on johtanut tahaamattomuutta aivokuolemaan johtanut tapahtumaa, koska elinluovuttajien puute on johtanut tahaamattomuutta aivokuolemaan johtanut tapahtumaa, koska elinluovuttajien puute on johtanut tahaamattomuutta aivokuolemaan johtanut tapahtumaa, koska elinluovuttajien puute on johtanut tahaamattomuutta aivokuolemaan johtanut tapahtumaa, koska elinluovuttajien puute on johtanut tahaamattomuutta aivokuolemaan johtanut tapahtumaa, koska elinluovuttajien puute on johtanut tahaamattomuutta aivokuolemaan johtanut tapahtumaa, koska elinluovuttajien puute on johtanut tahaamattomuutta aivokuolemaan johtanut tapahtumaa, koska elinluovuttajien puute on johtanut tahaamattomuutta aivokuolemaan johtanut tapahtumaa, koska elinluovuttajien puute on johtanut tahaamattomuutta aivokuolemaan johtanut tapahtumaa, koska elinluovuttajien puute on johtanut tahaamattomuutta aivokuolemaan johtanut tapahtumaa, koska elinluovuttajien puute on johtanut tahaamattomuutta aivokuolemaan johtanut tapahtumaa, koska elinluovuttajien puute on johtanut tahaamattomuutta aivokuolemaan johtanut tapahtumaa, koska elinluovuttajien puute on johtanut tahaamattomuutta aivokuolemaan johtanut tapahtumaa, koska elinluovuttajien puute on johtan

Sydänsiirrettä kuormittavat verenkertosairaudet sisältävät sekä immunologiset (mm. iskemia-reperfuusiovaurio) ja immunologiset (mm. alloimmuunireaktio) vastteet sekä immunosuppressiivisen lääkityksen sivuvaikutukset. Nämä edusattavat kroonisen hyljintäreaktion kehittymistä. Pitkääikaishyljinnän vallitsevina piirteinä ovat sydänsiirteen valtimoopuston kiitykykyyn ja diffuusia ahtautumioita, entenevä parenkymmen fibroitumisen sekä voimakas tulehdusfiltrointi sekä kudosnekoosi. Kylmäsäilytysajan pituus on merkittävämpi ensnauktojen kehittämiselle.

Viime vuosina on selvinnyt, että statiinin kolesterolivälitteisiä vaikutuksia laajempi.

Viime vuosina on selvinnyt, että statiinihoidon sydän- ja verenkiertosairauksiin sairastava pelataan ja kuolleisuutta vähentävä se on selvästi sen kolesterolivälitteisiä vaikutuksia laajempi. Statiinilääkäisyys liitetään usein immunosupressiivisen hoidon oheen elinsiirtoopitalalle, mikäli heillä ei tätä enne todettu ole käytössä. Statiinihoidon yhteydessä on todettu parantavan sydän- ja munuaissiirteiden kiitykykyyn sekä kolesterolisii vaikutuksia laajempi.


Koeasetelma vastaa kliinistä sairaalaatilannetta, jossa aivokuoleman jälkeen teho-osaston anestesiälääkäriitä tarjoutuu muutaman tunnin aikaikkuna hoitaa elinluovuttajaa ennen elinten irrotusvaihetta. Perustutkimustulosten pohjalta olemme käynnytä HUS:n eettisen
toimikunnan luvalla kliinisen randomisoidun tutkimuksen, jossa elinsiirteiden luovuttajia esilääkitään vastaavalla koeasetelmalla. Luovuttajan esilääkitys simvastatiinilla tarjoaa lupaavan hoitomahdollisuuden elinsiirtopotilailla ilman elinsiirteen vastaanottajaan kohdistuvia sivuvaikutuksia.
SAMMANFATTNING (SWEDISH SUMMARY)

Mikrovaskulär dysfunktion i hjärt- och njurtransplantat med ischemia-reperfusion skador

Långtidsprognosen för hjärttransplantationer är rätt dålig trots flera framsteg som tagits under de senaste åren. Hjärttransplantaten belastas såväl av blodomloppssjukdomar, immunförsvarrelaterade (bl.a alloimmun-reaktioner) och -orelaterade (bl.a sk. ischemia-reperfusion skador) reaktioner såsom biverkningar förorsakade av immunsuppressiv medicinering. Dessa faktorer befrämjar utveklingen av en kronisk lågtidsrejektionsreaktion som karakteriseras av artärgrenarnas accelererade förträngning i transplantatet. Kylförvarning är en av de viktigaste enskilda prognosfaktorena för funktionen av transplantatet.


I vår försökskonstellation användes råttan som modell för hjärt- och njurtransplantationer. Hjärttransplantat utsattes för 4 timmars kall- och 1 timme varmförvaring medan njurtransplantat utsattes för 16 och 1 timme, respektive förvarningstyp. Resultaten påvisar att nytta med transplantatdonorens statin förbehandling grundar sig främst på statinets skyddande inverkan på blodådrorna. Den kliniskt anpassade engångsdosen för simvastatin, 2h före lösgöring av hjärt- och njur transplantaten, förhindrade bildningen av stressfibrer i blodådrornas endotelceller, öppningen av endotelfögarna, ökning av blodådrornas permeabilitet samt perisytkontraktilitet och förstörningar i vävnadsperfusion. Våra resultat påvisar att simvastatin förbehandling av hjärt- och njurtransplantat, leder till en nedreglerad expression av gener som styr bildningen av fibros och inflammationsreaktion, nedsatt förträngning av artärgrenar i hjärttransplanten och en förbättrad prognos för långtidsrejektioner. Då transplantatdonorens simvastatinförbehandling kombineras med en metyLPrednisolonförbehandling, uppnås en effekt som både skyddar blodådor och lindrar inflammationsreaktioner, och därmed förbättrar hjärttransplantatets prognos under en akut rejektion.

Vår försökskonstellation motsvarar verkligheten i och med att i fall av patientens hjärndöd, kan förmedicinering tillämpas på donoren före lösgöring av transplantatet. Resultaten som uppnåtts genom denna basforskningen har tillämpats vid HUS till att starta ett kliniskt randomiserat försök, där transplantatdonorer förmedicineras i enlighet med vår forskningskonstellation. Simvastatin förmedicinering utgör ett lovande vårdalternativ, utan belastande biverkningar på transplantataceptorn.
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I dedicate this thesis to most important people of my life, my love Erika and our children Aurora and Adrian.

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