Myoblast transplantation and adenoviral VEGF-C transfer in porcine model of coronary artery disease

Tommi Pätilä

Department of Cardiothoracic Surgery

Helsinki University Central Hospital

Finland

Academic Dissertation

To be presented, with the permission of the Medical Faculty of

the University of Helsinki, for public examination in Auditorium 1,

Biomedicum Helsinki, University of Helsinki, Haartmaninkatu 8,

on February 27th, 2009 at 12 noon

Contents:

Abbreviations

ACC	American College of Cardiology
Ad	Adenovirus
AHA	American Heart Association
CABG	coronary artery bypass grafting
CoDe	coincidence detection
DMEM	Dulbecco's Modified Eagle's Medium
EDV	end-diastolic volume
ESV	end-systolic volume
HFNEF	heart failure with normal ejection fraction
ICD	intracardiac defibrillator
IVUS	intravascular ultrasound
LacZ	β-galactosidase
LCx	Left Circumflex coronary artery
LVAD	left ventricular assist device
LV	left ventricle
LVEF	left ventricular ejection fraction
PCI	percutaneous coronary intervention
PET	positron emission tomography
RAA	Renin-angiotensin-aldosterone
SPECT	single photon emission computed tomography
VEGF	vascular endothelial growth factor
18-FDG	18-flurodeoxyglucose

List of original publications

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

I   Pätilä T, Ikonen T, Rutanen J, Ahonen A, Lommi J, Lappalainen K, Krogerus L, Ihlberg L, Partanen TA, Lähteenoja L, Virtanen K, Alitalo K, Ylä-Herttuala S, Harjula A. Vascular Endothelial Growth Factor C–induced Collateral Formation in a Model of Myocardial Ischemia. J Heart Lung Transplant. 2006;25:206-13.

II   Ikonen T, Pätilä T, Virtanen K, Lommi J, Lappalainen K, Kankuri E, Krogerus L, Harjula A. Ligation of Ameroid-stenosed Coronary Artery Leads to Reproducible Myocardial Infarction – A Pilot Study in a Porcine Model. J Surg Res. 2007;142:195-201.

III   Pätilä T, Ikonen T, Kankuri E, Ahonen A, Krogerus L, Lauerma K, Harjula A. Multimodality Detection of Myocardial recovery after Ligation of Ameroid-Stenosed Coronary Artery. Scand Cardiovasc J. Submitted.

IV   Pätilä T, Ikonen T, Kankuri E, Uuutela A, Lommi J, Krogerus L, Salmenperä P, Bizik J, Lauerma K, Harjula A. Improved Diastolic Function

1. Abstract

Heart failure is a common and highly challenging medical disorder. The progressive increase of elderly population is expected to further reflect in heart failure incidence. Recent progress in cell transplantation therapy has provided a conceptual alternative for treatment of heart failure.

Despite improved medical treatment and operative possibilities, end-stage coronary artery disease present a great medical challenge. It has been estimated that therapeutic angiogenesis would be the next major advance in the treatment of ischaemic heart disease. Gene transfer to augment neovascularization could be beneficial for such patients.

We employed a porcine model to evaluate the angiogenic effect of vascular endothelial growth factor (VEGF)-C gene transfer. Ameroid-generated myocardial ischemia was produced and adenovirus encoding (ad)VEGF-C or β-galactosidase (LacZ) gene therapy was given intramyocardially during progressive coronary stenosis. Angiography, positron emission tomography (PET), single photon emission computed tomography (SPECT) and histology evidenced beneficial affects of the adVEGF-C gene transfer compared to adLacZ. The myocardial deterioration during progressive coronary stenosis seen in the control group was restrained in the treatment group.

We observed an uneven occlusion rate of the coronary vessels with Ameroid constrictor. We developed a simple methodological improvement of Ameroid model by ligating of the Ameroid–stenosed coronary vessel. Improvement of the model was seen by a more reliable occlusion rate of the vessel concerned and a formation of a rather constant myocardial infarction. We assessed the spontaneous healing of the left ventricle (LV) in this new model by SPECT, PET, MRI, and angiography. Significant spontaneous improvement of myocardial perfusion and function was seen as well as diminishment of scar volume. Histologically more microvessels were seen in the border area of the lesion. Double staining of the myocytes in mitosis indicated more cardiomyocyte regeneration at the remote area of the lesion.

The potential of autologous myoblast transplantation after ischaemia and infarction of porcine heart was evaluated. After ligation of stenosed coronary artery, autologous myoblast transplantation or control medium was directly injected into the myocardium at the lesion area. Assessed by MRI, improvement of diastolic function was seen in the myoblast-transplanted animals, but not in the control animals. Systolic function remained unchanged in both groups.

2. Introduction

End-stage coronary artery disease and heart failure cause significant burden to western society. Patients are disabled due to terrible chest pain or shortness of breath in minimal daily activities. When heart diseases are amongst the most common causes of death in western countries, these diseases present a state of ultimately weared out heart, with severe symptoms and a general lack of treatment choises.

End-stage coronary artery disease is defined as persistence of angina pectoris symptoms CCS class III and IV despite maximally tolerated conventional medical treatments and in-eligible coronary arteries to conventional revascularization procedure. Commonly, coronary disease is characterized by proximal stenoses of the main coronary vessels, rather easily dilated percutanously or bypassed surgically with modern techniques. However in end-stage coronary disease the coronary arteries are severely narrowed and diseased, and are beyond the most modern stenting procedures and surgical techniques. Many of these patients have already experienced multiple stentings and/or multiple coronary bypass procedures. These patients are systematically excluded from randomized trials of coronary bypass versus medical therapy and versus percutaneous techniques. Data on optimal management of this increasingly important and large patient subset are scarce (Schoebel and others 1997) .

Heart failure is a syndrome caused by the inefficiency of the heart to provide enough blood to tissues. Most commonly this is due to LV malfunction (Mann and Bristow 2005). The basic modern treatment is based on optimal medication according to structural disease of the heart and symptoms. Patients with refractory symptoms despite maximal medication are offered extraordinary measures, such as chronic inotropes, mechanical assist devises or surgery. Any of these provide a stable long-term result.

While cardiac transplantation provides best results for end-stage coronary artery disease and heart failure, the patients are often old and thus beyond transplantation programs. Also, graft shortage is a worldwide dilemma. Thus, alternative means of treating these patient groups should be developed (Hunt and others 2005).

One approach to cardiovascular ischemic diseases consists of augmenting neovasculariza-tion with the aid of recombinant growth factors or gene therapy. It has been estimated that therapeutic angiogenesis would be the next major advance in the treatment of ischaemic heart disease. Furthermore, autologous transplantation of precursor or stem cells to replace dysfunc-tional myocardium has emerged as a novel surgical alternative in the treatment of heart failure. In this study, our aim was to evaluate the duration of the biological effect of adenovirus medi-ated VEGF-C in ischemic porcine heart. We also modified the ischemic model based on the Ameroid constrictor, to provide more consistent coronary artery occlusion. Finally, we aimed to test the effect of autologous intramyocardial myoblast transplantation in ischemic and inf-arcted myocardium.

3. Review of the literature

Heart failure

Heart failure is a syndrome in which the heart is unable to pump enough blood for body's needs. Chronic low perfusion in tissues leads to neurohormonal changes, dyspnoea and fatigue - a state or syndrome called congestive heart failure. Heart failure is not an independent disease, but always a derivative of cardiac or extracardiac, congenital or acquired disease (Mann and Bristow 2005). In the 2001 report, the American College of Cardiology (ACC)/American Heart Association (AHA) introduced four stages in the development of heart failure (Table 1.). The first two stages recognize patients with factors that predispose them to the developing heart failure. Third stage includes patients with current or previous heart failure and a known heart disease. Fourth stage includes patients who have refractory heart failure despite optimal medical care and who may be suitable for focused treatments (Hunt and others 2001). New York Heart Association classification categorizes heart failure in four grades (I-IV) according to the degree of functional impairment conferred by the abnormality (Criteria Committee, New York Heart Association 1964).

In a large European study, prevalence was higher in men and increased with age from 0.9% in subjects aged 55–64 to 17.4% in those aged 85 (Bleumink and others 2004). Heart failure contained a three-year mortality rate 41% in patients <65 years and 66% for patients ≥65 years (Cleland and others 1999). The heart failure is primarily caused by coronary artery disease and is the consequence of previous myocardial infarction. Other common causes of heart failure are hypertension and valve diseases. Together these three etiological factors explain a major deal of all heart failures (Sutton 1990). The less common causes of heart failure are eg. cardiomyopathy, pericardial diseases, congenital malformations, lung vessel and lung diseases excessively loading right ventricle, and tumours of the heart.

The heart failure can be diastolic or systolic in nature (Aurigemma and Gaasch 2004). Accumulative evidence suggests that these phenomena cannot be entirely distinguished from each other, and the preferred term for diastolic dysfunction should be heart failure with normal ejection fraction (HFNEF). The proportion of patients with HFNEF in various studies ranges from 13-74%, major of studies reporting a value of 40% (Vasan, Benjamin, Levy 1995). However, many studies are compromised by variable definitions of heart failure and the precise threshold for what is considered to be a normal left ventricular ejection fraction (LVEF). Patients with primarily diastolic heart failure generally exhibit a concentric pattern of hyperthophic process and LV remodelling with a high ratio of wall thickness to chamber radius. On the other hand, systolic heart failure exhibits eccentric LV remodelling with increased chamber volume and slightly increased LV wall thickness (Devereux and others 2000). In systolic heart failure the ratio of mass to volume and LV wall thickness to chamber radius is decreased.

Stages of heart failure	
Stage	Symptoms
A	At risk for heart failure but without structural heart disease or symptoms
B	Structural heart disease but without signs or symptoms of heart failure
C	Structural heart disease with prior or current symptoms of heart failure
D	Refractory heart failure requiring specialized interventions

Table 1. Classification of heart failure according to ACC/AHA statement.

Medical therapy for heart failure

The current therapy for heart failure is mainly limited to the treatment of already established disease and is primarily pharmacological in nature. The introduction of angiotensin convertase enzyme inhibitors have changed dramatically the prognosis of heart failure. Renin-angiotensin-aldosterone system attempts to retain sufficient blood pressure and renal glomerular filtration in the low cardiac output state. Anyhow, the activation of this system has more deleterios effects than advantage on a long term. The inhibition of conversion of angiotensin I to angiotensin II restrains the pathophysiological effect of the latter. The same effect can be reached with angiotensin receptor I inhibitors. Also aldosterone inhibitors, such as spironolactone act to revert renin-angiotensin-aldosterone system disorder. The use of angiotensin convertase enzyme inhibitors or angiotensin reseptor blockers are recommended in all the ACC/AHA stages of heart failure. Stage B patients are additionally recommended beta blockers in selected patients. On the Stage C patients diuretics, digitalis, aldosterone inhibitors and hydralazine or nitrates are recommended. On the patients with a refractory heart failure despite optimal medical care staged as D, extraordinary measures such as chronic inotropes, mechanical support, heart transplantation or surgical treatment can be offered (Hunt and others 2005).

Surgical treatment for heart failure

When assessing the surgery as an option for heart failure, there are no guidelines or clear algorithms as to which operation would suit an individual patient the best. Coronary surgery may improve the contractility of stunned or hibernating myocardium in 60% to 70% of cases (Elefteriades and others 1993; Langenburg and others 1995). A critical mass of reversible ischemic myocardium must be present to attain a recovery in the left ventricular function. As heart failure progresses, changes in the left ventricular configuration cause the papillary muscles to stretch out of shape. Mitral valve regurgitation repair in the case of inappropriate approximation improves prognosis in heart failure patient population, but the long-term results are unsatisfactory (Ngaage and others 2008). Valve replacement surgery might improve exercise tolerance and quality of life in patients with heart failure with a concomitant other valve disorder. A number of studies have shown that heart failure symptoms can be improved with a biventricular pacing or cardiac resynchronization therapy (Bristow and others 2004; Cleland

and others 2005). Left ventricular surgical restoration by excluding the noncontractile portion of the anterolateral left venricle with or without revascularization or mitral valve correction, improves left ventricular function and decelerates the remodelation process, when applied to appropriate patients (Athanasuleas and others 2004). Prosthetic devices for ventricular remodelling surgery in which the heart is wrapped in a mesh-like devise, have been introduced with to some extent positive results (Acker 2004). Implantation of left ventricular assist devises has been shown to be expensive with poor long-term results and significant complications. Two years after implantation of such devise, there are almost no survivals, although the patient population receiving the pumps enjoy improved quality of life compared to the control patients (Rose and others 2001). Batista procedure was introduced during the mid-1990s for patients with heart failure and dilated LV. This operation consists of removing a flap of heart muscle from the LV to shrink the size of the ventricle and improve its geometry, thus improving the efficiency of cardiac function.of patients with severe heart failure. In a series of 59 patients with severe heart failure and left ventricular dilation. 25% improved, 33% of the patients rapidly deteriorated, and the remainder had only transient improvement in cardiac function after the surgery, after which deterioration again set in.   After these results, general interest in this operation rapidly diminished (Starling and others 2000). Dynamic cardiomyoplasty is a method in which autologous latissimus dorsi muscle is wrapped around the heart as a pedicle graft. Stimulation of the graft can be synchronized with cardiac systole. Mortality in this operation has been descibed less than 10% and 80-85% or the survivals show improvement in NYHA class (Salmons 2008). For patients with end-stage heart failure the only relevant surgical option would be heart transplantation (Hunt 2006). Anyhow, transplantation is restricted by organ shortage and immunosupression. Also the contraindications of heart transplantation rule out a major part of the end stage heart failure patients. Thus, although heart transplantation is of major importance for an individual recipient, its overall impact is limited. The use of xenografts is an alternative option which would resolve many problems concerning heart transplantation, but it is still beyond a clinical option at present (McGregor and others 2005).

Experimental models of heart failure

Pressure overload of the heart can be used to study the myocardial response to increased work. In the creation of right ventricle overload, the technique of pulmonary trunk banding is similar to in the Blalock-Hanlon operation in transposition of great arteries in infants. This maneuver produces hypertrophy of the right ventricle and failure takes place only after severe degrees of constriction. Similar models with supravalvar aortic constriction produces pressure loading of LV (Henderson and others 2007). LV pressure loading can also be produced pharmacologically by administration of corticosteroids. Occlusion of renal artery or unilateral nephrectomy and contralateral renal artery clipping leads also to hypertension and eventually left ventricular hypertrophy.

Volume overload can be performed at simplest by intravenous infusion of excess fluids. This kind of model can be used to study pulmonary congestion. Surgical instrumentation of aortocaval fistula leads to right side volume load and this kind of model has been produced in rat and dog. Similarly a chronic right side volume overload can be created by causing an atrial septal defect. LV volume overload can be produced by creation of aortic valve incompetence.

These situations provide a model of heart dysfunction clinically relevant to surgical conditions (Ryan and others 2007).

Myocardial infarction is the most employed model of heart failure. This kind of model resembles clinical situation, but the amount of the infarction appears to be a challenge. Lesser infarction leads to compensatory changes, and excessive myocardial loss causes cardiogenic shock and death. Coronary occlusion can be extravascular or intravascular in nature (Schmitto and others 2008).

Cardiomyopathy occurs spontaneously in Syrian hamsters and turkeys, so that heart failure can be produced congenitally by specific breeding programs. Primary myocardial disease can also be produced by a variety of compounds toxic to myocardium. Furazolin, adriamycin (Lu and others 2008) and barbiturates have been used successfully to produce heart failure. Several other compounds such as catecholamines and alcohol have been used for cardiac depression. Also chronic hypoxia and sustained rapid atrial pacing lead to myocardial dysfunction (Moe and Armstrong 1999; Smith and Nuttall 1985).

Concept of heart regeneration

Zebrafish has been demonstrated to response to apical heart amputation by regeneration of LV with only a small deposits of collagen. After a removal of 20% of the myocardium a new ventricular wall of compact myocardium was created by cardiomyocyte proliferation. It appeared, that these proliferating cells migrated from the adjacent healthy epicardium (Poss, Wilson, Keating 2002). Anyhow, no such regenerative healing has proven to exist in mammalian heart, in which myocardial infarct heals by means of extensive scarring (Robey and Murry 2008).

Human heart is a post mitotic organ responding mainly by myocyte hypertrophy, cellular reorganisation and fibrosis to excessive load and hypoxic trauma. According to this dogma, human hearts do not regenerate. Anyhow, evidence of myocyte proliferation has been observed in histological studies of hearts of end stage failure, where 140 mitosis per 1 million cells was observed (Kajstura and others 1998). Control hearts showed one tenth the amount of mitosis. In another study, myocardial samples were collected of 13 patients, who had died in myocardial infarction 4 to 12 days previously. In these samples, mitotic myocyte nuclei was found in 4 percent of adjacent to the infarction and 1 percent in the remote areas (Beltrami and others 2001). Mitosis is a process of one hour duration and the authors propose, that the rather high mitotic index is evidence of the clinically significant cellular hyperplasia.

Calculations of the rate of apoptosis added to the new information of proliferation raised suspicion of the slow regeneration and cell renewal in the human heart. It has been proposed that part of this phenomenon can be explained by artifacts (Schaper, Elsasser, Kostin 1999). More profound analysis of the replicating myocytes has shown, that the size of the myocytes in mitosis is significantly smaller than the size of an average cardiomyocyte and the origin of the new cell might be extracardiac. Y-chomosome positive cardiomyocytes in male heart transplant recipients, who had female donors, lead to high suspicion of external source of these new cardiac cells (Quaini and others 2002; Bayes-Genis and others 2002). The possibility of migration of the cells from the allograft remnants has been challenged by groups, who have transplanted extracardially different types of marked cells and reported the engraftment of these cells to damaged heart. Only a small fraction of these cells differentiate into cardiac myocytes (Jackson

and others 2001). They mainly demostrate blood cells, predominantly granulocytes, but of the part of the cells differentiating into cardiac cells have resembled endothelial cells in many studies (Balsam and others 2004; Nygren and others 2004). The results of the studies in this area of research are complex and yet unresolved.

Cell therapy for heart failure

Cell-based cardiac repair offers the promise of alleviating heart injury by reconstituting or maintaining cardiac specific tissue (Orlic and others 2001). The preliminary studies were conducted with dedicated cells such as myoblasts (Chiu, Zibaitis, Kao 1995), but soon after the field expanded to an array of cell types including bone marrow cells (Bittner and others 1999), endothelial progenitors (Asahara and others 1997), mesenchymal stem cells (Pittenger and others 1999), resident cardiac stem cells (Beltrami and others 2003), and embryonic stem cells (Westfall and others 1997). There has been numerous preclinical studies showing improvement in animal cardiac failure models, yet the mechanism of the improvement has remained obscure. Anyhow, the hypothesis of cardiac failure reversal or prevention has gained widespread attention and early-stage clinical trials have been launched (Assmus and others 2007; Chen and others 2004; Janssens and others 2006; Lunde and others 2006; Schachinger and others 2006; Wollert and others 2004).

Routes for cell delivery

Transvascular route is suitable for patients undergoing percutaneous coronary interventions (PCI). The bone-marrow derived cells can be infused during the balloon inflation, when coronary blood flow and thus the cell washout is minimal. Such intracoronary infusion would also permit to deliver the cells to a certain area. In the setting of myocardial infarction, the activation of adhesion molecules and chemokines enhance the engraftment of the transplanted stem cells (Strauer and others 2002). Thus, the cell transplantation at the time of myocardial infarction and reperfusion seem a reasonable option. Intravenous stem cell transplantation is an additional option in the case of acute myocardial infarction, but the amount of the cells homing to the heart is low (Kocher and others 2001a). Since skeletal myoblasts do not extravasate and may cause microembolization after coronary infusion, the intravascular route in not a possible option. The transplantation of myoblast cells in to the heart requires direct cell injection to the myocardium or other method of topical application.

Direct injection of the cells into the left ventricular wall can be performed via transepicardial or intravascular route. Intravascular direct myocardial injections can be performed endocardial or transcoronary vein injections (Siminiak and others 2006). Catheter based transendocardial injection is performed using a needle catheter directed perpendicular to the inner surface of the target area an electromechanical mapping of the endocardial surface (Rutanen and others 2004c). The delivery of the cells by direct intraventricular injections has been proven to be feasible, but the method has certain difficulties. The LV as a moving target, especially post-infarction thin wall areas, might appear technically demanding. Also the back-flush of the cells from the puncture holes is probable. Catheter based cell delivery through coronary veins

consists of a catheter based endovascular system incorporating an intravascular ultrasound (IVUS) source and an extendable needle. After placing the catheter system into the target coronary vein, the IVUS is used to orient the direction of the needle according to pericardium, ventricular myocardium and the corresponding artery as landmarks. The pre-shaped Nitinol needle is oriented parallel to the ventricular wall (Sherman and others 2006).

Myoblast cells

Skeletal muscle is composed of multinucleated cells (muscle fibers), which may be 10-100μm thick and up to 15 cm long. These cells consist of transverse striations due to periodic alternation of isotropic and anisotropic bands. The nuclei of these cells lie immediately beneath of the cell surface and are scattered in the direction of the long axis of the muscle fibers. Skeletal muscle nuclei have lost the ability to divide and can be considered simply as transcriptional units. Already at 1961 Mauro et al reported seeing under electronic microscopic view, mononucleated cells under the basement membrane of the muscle fiber but not fused with the main muscle fiber (Mauro 1961). Mauro suggested these cells to be dormant myoblasts that failed to fuse with other myoblasts and are ready to activate, when the main multinucleate cell is damaged. They named the cells as satellite cells. This highly speculative suggestion turned out to be right. The potential role of satellite cells in repair has been under investigation in several morphological and functional studies. The simplified description of satellite cell has been challenged recently, indicating that under this name is a heterogeneous hierarchical population of cells. This population is composed of a small number of satellite stem cells and a larger number of committed myogenic progenitors (Kuang and others 2007). There is also evidence, that rare subpopulations of bone marrow cells (Ferrari and others 1998; Gussoni and others 1999), a minority of circulating cells (Torrente and others 2003), or cells emanating from blood vessels (Sampaolesi and others 2003) can also adopt myogenic fate under certain circumstances. The descendants of activated satellite cells are called myogenic precursor cells or skeletal myoblasts. These cells are able to undergo multiple rounds of division prior to terminal differentiation and fusion to form multinucleated myofibers (Le Grand and Rudnicki 2007).

Myoblast transplantation therapy

The finding of myoblast and satellite cells aroused a high hope for possible clinical applications. The myoblast cells have several advantages. Satellite cells are able to differentiate and fuse to augment existing muscle fibres and to form new fibres. These cells are involved in the normal growth of muscle, as well as regeneration following injury or disease. In undamaged muscle, the majority of satellite cells are quiescent; they neither differentiate nor undergo cell division. In response to mechanical strain, satellite cells become activated. Activated satellite cells initially proliferate as skeletal myoblasts before undergoing myogenic differentiation. The main advantages of the myoblasts include autologous origin, which overcomes the problems associated with immunosuppression. The cells are easily harvested, they are rather easily purified and they show substantial proliferative potential allowing significant increase in cell number. Myoblasts are resistant to ischemia, which theoretically enhance the engraftment at a transplantation site of less vascularized tissue (Menasche 2008). Although rhabdomyosarcoma has

been proposed to be origin of satellite cells, the advanced state of differentiation in myoblast or myogenic cells eliminate the possibility of tumorigenity (Tiffin and others 2003).

Studies in mdx mouse, a mouse strain lacking muscular dystrophin as in Duchenne muscular dystrophy, showed strong potential of myoblast transplantation (Partridge and others 1989). However, despite the promising results in mouse model, clinical trials conducted in humans showed very limited success (Huard and others 1992). The disappointing shift from rodent model to man, raised questions of the drawbacks of the myoblast transplantation. Indeed, many adverse events have been described following myoblast injection to muscle. Beauchamp et al showed, that massive cell death ensue the myoblast transplantation to mouse mucles, the survival being less than 1% after four days (Beauchamp and others 1999). In this study, Radioactively labelled male myoblast cells were injected to female mouse. Despite the radiolabels and Y-chromosomes diminished initially linearly and substantially during time, at the day four the ratio of the radiolabels and Y-chromosomes showed, that the surviving population of the transplanted cells were dividing. The investigators were able to find within the transplanted cells, a population of in vitro slowly dividing cells, which survive and undergo rapid proliferation in vivo after transplantation. This finding was later supported by Montarras et al, who were able to isolate by flow cytometric means satellite cells (nongranular, Pax7+, CD34+, CD45−, Sca1−) from Pax3GFP/+ mouse diaphragm. The ability of these cells to contribute to tissue reconstitution in mdx mouse was not increased by in vitro proliferation. Conversely, the amplification of cell number seemed not change the tissue reconstitution rate of a cell population.

The culture of muscle progenitor cells before grafting markedly reduced their regenerative efficiency in a way, that the culture expansion yielded the same amount of muscle as the number of cells from which the culture was (Montarras and others 2005). Limited proliferative capacity of somatic cells explained by telomerase shortening includes satellite cells (Decary and others 1997). Telomere shorten at each cell division and once the telomere becomes too short, a DNA damage signal is generated triggering the p53 expression and proliferative senescence. The poor dispersion of the injected cells might also be part of the undesirable regenerative effect of the myoblasts (Skuk and Tremblay 2003).

Myoblast cell therapy for heart failure

The mechanisms of cell transplantation to improve the function of damaged myocardium has several explanations. The electromechanical coupling with the recipient heart and the transplanted cells participating in a functional syncytium with the host myocardium is most obvious, but improbable explanation. The engrafted myoblasts form large myotubes, which possibly contract according to the host myocardial cells due to the mechanical stress. The lack of connexin-43 expression of the myoblasts prevent the cells to be electrophysically connected to surrounding cardiomyocytes (Reinecke and others 2000), but the myoblasts retain their excitability and can generate action potentials in the presence of field stimulation (Leobon and others 2003). Anyhow, the detected improvement of systolic function may be mediated by paracrine factors secreted by the engrafted cells (Kinnaird and others 2004). Enhanced formation of blood vessels has been detected after cellular transplantation and increased expression of variety of growth factors observed. In a hibernating or stunned myocardium it has been noted, that improved perfusion promotes the recovery of systolic function. The myoblasts may

fuse with cardiomyocytes forming cardiac-skeletal hybrid cells, as a rare event (Reinecke and others 2004). In vitro these fused cells resembled morphologically skeletal myotubes, while in vivo they were similar to cardiomyocytes.

Another explanation would be the limitation of postinfarction LV remodellation. Such a mechanism is dependant on the timing of the cell transplantation, because notable reversal of severely remodellated LV is not likely, whereas early postinfarction cell grafting would inhibit the post-infarction expansion (Formigli and others 2007). Skeletal myoblast transplantation has also been associated with a significant attenuation of matrix metalloproteinase-2 and -9 up-regulation in a mouse model thus affecting to the degree of fibrosis (Murtuza and others 2004).

Human studies of myoblast therapy for heart

Skeletal myoblasts were the first form of myocardial cell replacement therapy to enter the clinical arena in 2000 (Menasche and others 2001). Menasche et al reported a case of autologous myoblast transplantation in a patient undergoing coronary artery bypass grafting (CABG) surgery. The patient suffered severe heart failure with LVEF 21% and cardiac output 1 L/min. They injected 800 million cells at the free inferior wall at the area of infarction. Five months after the surgery, the symptoms had relieved significantly and echocardiography showed improved systolic thickening at the site of transplanted cells. Positron emission tomography showed improved tracer uptake at the inferior wall. LVEF was measured 30% at five months after the surgery. The patient died 17,5 months after the operation due to stroke caused by acute proximal subclavian artery occlusion. At autopsy, histological analysis showed presence of multinucleated myotubes embedded within the fibrosis measuring up to 4mm in length without any inflammation or fibrosis or neovascularization. These cells were aligned to the same direction as the adjacent cardiomocytes (Hagege and others 2003).

Several feasibility and safety pilot studies followed. In Paris, ten patients received myoblast transplantations during CABG surgery. All the patients had severe left ventricular dysfunction with LVEF less than 30% and a non-viable post-infarction scar area detected by dobutamine echocardiography, 18-fluorodeoxyglucose (FDG) PET. These patients received an average of 871 x 106 autologous myoblasts at the area of infarction. One patient died unrelated to the cell transplantation. All the other patients had uneventful recovery. Four patients out of ten suffered sustained   ventricular tachycardia and were implanted with an internal defibrillator. Because of the nature of the heart disease itself, the etiology of the arrhythmias remained obscure. Echocardiographic analysis showed that 63% of the cell-implanted scar segments (14 out of 22) demonstrated improved systolic function (Menasche and others 2003).

Herreros et al treated twelve patients with simultaneous myoblast transplantation and CABG. The cells were implanted at akinetic or dyskinetic areas of LV via direct transepicardial injections. Echocardiography revealed a marked improvement in regional contractility in those cardiac segments treated with skeletal myoblast and 8F-FDG PET studies showed a significant increased in cardiac viability in the infarct area 3 months after surgery. The mean improvement in the LVEF was 18% (Herreros and others 2003).

In a multicenter study conducted by Dib et al., 24 patients received myoblasts in a direct transepicardial injections at a simultaneous CABG operation (Dib and others 2005). Additional 6 patients, who received left ventricular assist device (LVAD) as a bridge to transplantation had

myoblast injections. The amount of the myoblasts in the CABG group was 10-300 X 106 in a dose escalating manner and the dose in the LVAD group was a fixed 300 X 106. The main end point in the study was the appearance of adverse effects. In the LVAD group also the engaftment of the myoblasts were assessed in histological studies. For both the CABG and LVAD groups, the transplantation procedure was clinically well tolerated, and the myoblasts were delivered successfully. No deaths or arrhythmias occurred during surgery or injection of the cells. Minimal bleeding from the injection sites was seen on occasion. Four deaths occurred during the four year follow-up period: 3 in the LVAD group and 1 in the CABG group. None of them were deemed related to the myoblasts or cell transplantation procedure. There was improvement in viability detected by MRI and PET in the area of the myoblast transplantation in the CABG group. But none of the hearts receiving less than 300 x 106 cells showed improved viability. The mean baseline LVEF, measured by echocardiography, for the CABG patients was 28% at baseline and 36% at 24 months' follow up. The average end-diastolic volumes decreased from an average baseline 187 mL to 144 mL at 24 months. Because this procedure was combined with bypass surgery in the absence of a control arm, the improvement in LVEF, LV dimensions, and LV volumes, as related to cell transplantation, are unknown. In the hearts in the LVAD group areas of surviving myoblasts were seen in trichrome-stained sections and confirmed by use of the skeletal muscle-specific myosin heavy chain antibody, MY-32. No data of the number of the myoblasts surviving transplantion was obtained (Pagani and others 2003).

Smits et al. used endoventricular NOGA mapping and injection system to percutaneously transplant autologous myoblasts into an area of postinfarction injury (Smits and others 2003). Only five patients were enrolled in the study and the small sample size and the lack of control group restrains making further conclusions of the efficacy of the procedure. Anyhow, this study shows feasibility of the approach and the safety from a procedural point of view. An increase in the LVEF was observed in the LV-cinegraphy, but not in SPECT or magnetic resonance imaging (MRI). A subanalysis of this study, where additional five patients were enrolled (total n=10), consisted of regional and global LV function assessment by two-dimensional echocardiography with dobutamine infusion and tissue Doppler imaging. These studies showed an improvement in the systolic velocity at the myoblast transplanted LV wall and improved global LV function during low-dose dobutamin infusion, indicating an improvement of contractile reserve (Biagini and others 2006).

In another study performed by the same group, a sole procedure of transendomyocardial injections of autologous myoblasts were performed. At six months follow-up, an increased LVEF and cardiac output, a reduction of systolic LV volume and a trend towards improved stroke work were observed. These hemodynamic improvements were confirmed at one year after the myoblast transplantation by pressure-volume loops analysis, where significant increase in stroke volume, contractility and of diastolic stiffness was seen (Steendijk and others 2006).

In the first multicentre study of myoblast transplantation, the safety and efficacy of percutaneous transendocardial skeletal myoblast injection as a sole procedure in congestive heart failure patients was assessed. 15 patients were enrolled in the study. The mean LVEF was 34.4±10.3 and a mean 6±4 years had passed after myocardial infarction .The patients received 216±119 x 106 cells via transendocardial route with NOGA or fluoroscopy guided injection catheter. After treating the first 6 patients, the protocol was amended because of a sudden death due to arrhythmia. The additional patients were required to receive an intracardiac defibrillator

(ICD) prior to the procedure. After 1 year follow-up 13 patients were alive. Stress echocardiography showed improvement at rest and under low dose dobutamine stress, but there was no change in the LVEF (Smiths and others).

POZNAN-trial was similarly a catheter-based study, but the injection route was via cardiac veins (Siminiak and others 2005). Dobutamine stress echocardiography was performed to screen patients of a non-viable transmyocardial scar. Ten patients were enrolled in the study. Nine out of 10 patients received the myoblast transplantation according to the study plan. The patients received up to 100 x 106 cells within 2-4 injections. In one patient, technical issues concerning a valve at the bifurcation of the great cardiac vein prevented the treatment injections. After 6 months the NYHA class improved in all nine patients and all subjects were in class I during follow-up. Ejection fraction evaluated independently by two blinded experienced investigators increased 3–8% in six out of nine cases, and no change in the LVEF, despite improvement in the NYHA class, in the remaining three patients was observed.

All these aforementioned studies are not randomized and they lack a decent control group. The first phase II trial, Myoblast Autologous Grafting in Ischemic Cardiomyopathy trial evaluated the effect of autologous skeletal muscle myoblasts in patients with chronic heart failure who are undergoing coronary bypass. Inclusion criteria consisted of a history of myocardial infarction LVEF 15-35% and planned for CABG operation. The primary endpoint of the trial was improvement in LVEF observed in echocardiography and the secondary endpoint was 15% difference in 1-year major adverse cardiac events. The primary bioactivity endpoint was recovery of the wall motion in the areas of infarction and the myoblast replacement therapy. Secondary bioactivity endpoints were global LV function and volumes, Doppler tissue imaging sub-study, PET sub-study (Menasche and others 2008a) and viability and angiogenesis evidence in the engrafted region. The trial was originally designed to enroll 300 patients but was stopped early, with an intended enrollment of 120, after the data safety and monitoring board determined that the study was unlikely to show a benefit of treatment. Ultimately, only 97 patients with ischemic heart failure were actually randomized at 24 European centers. The low-dose group (n=33) received approximately 400 x 106 myoblasts delivered to 30 locations within and around the infarct site during the CABG procedure; the high-dose group (n=30) received 800 X 106 cells. Placebo group consisted of 34 patients. All the patients were implanted an ICD. 3 patients died after the cell transplantation and two randomized patients died awaiting the surgery without cell implantation. None of the deaths were considered to associate with the myoblast transplantations. LVEF was increased by 3% in the high-dose patients, compared to a 2% increase in the low-dose arm, and no change in the placebo arm (p=0.04 for high-dose compared to placebo; p=ns for low-dose compared to placebo). Also, LV end-diastolic volume was significantly improved in the high-dose arm compared to placebo (p-0.006), but not in the low-dose arm. LV end-systolic volume decreased by 18% in the high-dose patients, compared with a 3% reduction in the placebo patients (p=0.008). The investigators concluded that feasibility of autologous myoblast grafting was demonstrated. There were no serious adverse events in the high or low dose myoblast groups. There was an absence of significant improvement in regional or global contractility, but some evidence for reversal of remodeling.

CAUSMIC (First United States Randomized Controlled Trial Utilizing 3-Dimensional Guided, Catheter-Based Delivery of Autologous Skeletal Myoblasts for Ischemic Cardiomyopathy)- study was presented by Dib in the American College of Cardiology 56th annual meet-

ing in 2007 (Dib and others 2007). It showed improvement in NYHA class in the myoblast transplanted patients. A total number of 23 patients were enrolled in a randomized, but not blinded assessment had transendocardial injections of 30 – 600 x106 myoblasts performed with the NOGA system. The primary endpoints included feasibility and safety of the NOGA transplantation method. The functional efficacy was assessed by quality of life measures (NYHA class and Minnesota living with heart failure questionnaire), the viability by NOGA electromechanical mapping, and the function by echocardiography. Inclusion criteria consisted of previous heart failure, a nonviable scar and heart failure with a NYHA class II-IV. There was a significant improvement in the NYHA class in the myoblast-transplanted patients, but not in the control group of maximal medical treatment. There was a suggestive decrease in the ventricular volumes in the myoblast-transplanted patients, when the volumes increased in the control patients. According to the presented data, FDA approved a larger phase II randomized, double-blind, placebo-controlled multicenter study of 160 patients.

Ventricular arrhythmias after the myoblast transplantation

Episodes of ventricular tachycardia and fibrillation have been noted after the myoblast transplantation procedure. Because the studies in humans so far has boon non-randomized, the patient populations have been small and the heart disease has arrhythmogenic nature itself, it is difficult to show the direct causality. Anyhow, in the study of menasche, 4 out of 10 patient had sustained ventricular tachycardia and had ICD implanted (Menasche and others 2003). Furthermore, in the study of Smits et al., one patient died suddenly 9 days post procedure. Another patient (ICD patient) survived an electrical storm 12 days post procedure, but died 2 days later due to cardiogenic shock. Two other non-ICD patients received an ICD because of observed ventricular arrhythmias (Smits and others 2006). It remains unknown whether these events are directly related to the cell injections. In the MACIC-trial there was considered no myoblast associating significant arrhythmogeninity (Menasche and others 2008a) and in the FDA-approved forthcoming study planned by Dib et al., no prophylactic ICD implantation is needed (Dib and others 2007).

End-stage coronary artery disease

Coronary artery disease (CAD) is a leading cause of death in the western world (Rosamond and others 2008). Despite advances in the pharmacological and interventional treatment, a portion of the patients have diffuse coronary disease with small distal vessels unsuitable for intervention with significant symptoms with maximal medical therapy. In the western world, when the population ages, the amount of these patients may increase (Mukherjee and others 1999).

End-stage coronary artery disease is defined as persistence of angina pectoris symptoms class III and IV despite maximally tolerated conventional medical treatments and ineligible coronary arteries to conventional revascularization procedure. The function of the LV should be normal or near normal. Pharmacological treatment should be based on the three mainstays: nitrates, betablockers and calcium antagonists. Also a recent angiogram should be evaluated

to avoid withholding a possible CABG or PCI (Schoebel and others 1997). Alternative means of improving blood flow to the heart in these patients should be developed. Novel approaches in patients who have end-stage coronary artery disease should decrease anginal symptoms and increase functional capacity. Also increased life expectancy would be desirable.

Several alternative approaches to end stage coronary heart disease have been introduced. Intermittent urokinase therapy has been introduced as an anticoagulant approach. A dose of 500 000 international units three times a week for a period of 12 weeks decreased symptoms 70% compared to a control group of smaller dose (Leschke and others 1996). The possible mechanism of action was proposed to be dependent of rheological blood properties mediated by fibrinogen reduction, thrombolysis of non-occlusive subclinical thrombi, and regression of atherosclerotic plaques. In neuromodulation stimulation of vibratory efferent nerves or spinal cord would be effective in relieving angina pectoris symptoms (Mannheimer and others 1982). Several studies demonstrated improved symptom control, reduced nitrate usage, increased exercise tolerance and extended walking times to ischemia in CAD (Moore and Chester 2001). The nature of the treatment makes it rather impossible to randomize the treatment of the patient, and the placebo effect is difficult to rule out.

External enhanced counterpulsation (EECP) is based on a system, where compressed air is conducted to cuffs wrapped around patients lower extremities in a sequence synchronized with the cardiac cycle (Arora and Shah 2006). The EECP has claimed to improve symptoms and decrease long-term morbidity via several mechanisms, including improvement in endothelial function, promotion of collateralization, enhancement of ventricular function, improvement in oxygen consumption, regression of atherosclerosis, and peripheral training effects similar to exercise (Manchanda and Soran 2007). The first randomized study of EECP was presented by Arora et al (Arora and Shah 2006). In this study, CAD patients were randomized with similar patient group with similar non-effective pulsation system. EECP reduced angina in the treatment group and extended time to exercise-induced ischemia in patients with enhanced external counterpulsation. Recent evidence also suggests that ECCP may be applicable also for heart failure.

Transmyocardial (laser) revascularization (TMR) has been studied extensively. This technique consists of mechanical force to create small holes through the left ventricular wall. In a non-randomized multicenter trial of 200 end stage coronary patients with a sole therapy of TMR, the treatment provided angina relief, decreased hospital admissions, and improved perfusion assessed by single photon emission computed tomography. The procedure was performed via left anterolateral thoracotomy and perioperative mortality was 9% (Horvath and others 2005). All in all, six prospective randomized clinical trials have been performed with transmyocardial laser revascularization (Aaberge and others 2000; Allen and others 1999; Burkhoff and others 1999; Frazier, March, Horvath 1999; Jones and others 1999; Schofield and others 1999). 5 studies out of 6 have been performed using percutaneous method. All of the transmyocardial laser revascularization and 4 of the percutaneous myocardial revascularization studies showed a significant improvement in angina class. The results concerning improved survival, increased exercise tolerance, enhanced LVEF, and improved myocardial perfusion were less definitive. It has been stated, that TMR would have significant potential for morbidity and mortality (Tasse and Arora 2007). In the recommendations of the Society of Thoracic Surgeons for use of TMR (Edwards and Ferguson 2004), it is recommended as a sole therapy for patients with an LVEF

greater than 30% and CCSAS class III or IV angina that is refractory to maximal medical therapy, in the case when reversible ischemia of the left ventricular free wall is not amenable to revascularization. In a database study of 3,717 patients, mortality rate of 6.4% for TMR as a sole therapy was observed. Operative risks were higher among patients with recent myocardial infarction, low LVEF and unstable angina was observed (Peterson and others 2003).

Cardiac transplantation provides best results for end-stage coronary artery disease, but graft shortage is a worlwide dilemma. Furthermore, the patients are often old and thus beyond transplantation programs.

Porcine models for coronary artery disease

Different species has been used in an attempt to produce an animal model imitating human occlusive coronary artery disease. These models have been produced to study the physiological changes in heart in a condition of inadequate coronary blood supply. The physiological changes include effort ischemia, collateral vessel formation, hibernation, stunning, and microvessel dysfunction (Hughes and others 2003). Chronically stunned myocardium describes myocardial regions with persistent dysfunction despite normal basal coronary blood flow (Fallavollita, Perry, Canty 1997a; Fallavollita, Perry, Canty 1997b). This phenomenon of persistent postischemic dysfunction is considered to be a derivative of repetitive episodes of stress-induced ischemia in a state of insufficient coronary flow reserve. In the case of hibernation, there is persistent myocardial dysfunction with insufficient coronary flow at rest. Although these phenomena can be observed distinct, even so that specific characteristic structural alteration can be distinguished, in clinical situation there is much overlapping (Hughes and others 2001a). In a study comparing pre-existing collateral vessels between different mammalian species, a wide spectrum of pre-existing collateral network was demonstrated to exist between various mammalian species (Maxwell, Hearse, Yellon 1987). This study showed, that porcine heart has very sparsely arranged collaterals in the case of non-ischemic heart, similar to human. Conversely, this study demonstrated that the dog, which has been commonly used species in studies of myocardial ischemia, has a variable and often substantial collateral circulation network. These existing small vessels are able to provide up to 40% of normal flow to the myocardium distal to an acutely occluded coronary artery. As a result, the porcine gained popularity as species in coronary disease modelling. Furthermore, the comparison of macroscopic anatomy of porcine to human heart has showed great similarity, especially the coronary anatomy (Crick and others 1998a).

There has been several different methods of producing impaired coronary flow in ischemic porcine models. The Ameroid constrictor model has been the most used model of chronic ischemia. The Ameroid constrictor consists of a slowly swelling plastic ring enclosed in metal cover. The plastic ring is shaped of formaldehyde dried milk casein, which slowly absorbs water. When applied around an artery, the metal cover forces the plastic to swell towards the vessel. Vessel occlusion ensues within following weeks, although somewhat imprecise occlusion has been documented. Other methods include use of fixed stenosis (Chen and others 1997; Fallavollita, Perry, Canty 1997a; Fallavollita, Perry, Canty 1997a; Lai and others 2000) and hydraulic occluder (Bolukoglu and others 1992).

Porcine has also similarity in cardiac physiology compared to human, with the distribution of the coronary artery blood supply, including a typically right-dominant coronary system. Also the cardiac conduction system is very similar to humans (Swindle and others 1986). Likewise, the heart size-to-body weight ratio (0.005) for the typical 30-kg pig used in most laboratory studies is identical to that of humans (Hughes 1986). Finally, the swine heart is similar to that of humans from a metabolic standpoint as well. Principally nonesterified fatty acids are the main substrate under non-ischemic accounting for up to 80% of myocardial energy production (Abdel-Aleem and others 1999). During ischemia, the β-oxidation of fatty acids reduces, and the use of glucose increases.

Therapeutic angiogenesis

Therapeutic angiogenesis is an experimental concept for the treatment of ischemia of an end-organ due to occlusive vascular disease. It involves activation of vessel growth in a situation, when conventional revascularization procedures are not amenable. Three different processes may involve in the growth of the new blood vessels: vasculogenesis, arteriogenesis, and angiogenesis (Ferrara and Alitalo 1999; Ware and Simons 1997). Vasculogenesis is the process of vascular development during embryogenesis (Beck and D'Amore 1997). Arteriogenesis refers to the growth of vessels containing all the three layers of the artery wall. Arteriogenesis is dependant of shear forces, and the substrates of arteriogenesis are pre-existing collateral arterioles. Angiogenesis is the process of formation of new small capillaries consisting of endothelial cells (Ware and Simons 1997). The process of blood vessel growth occurs in a wide spectrum of different physiological and pathophysiological processes. Inflammation, tissue ischemia, hypertrophy, and wound healing are among other biological states, when new blood vessel growth has been shown to develop.

Several biologic mechanisms has been described as causal to arteriogenesis, such as endothelial cell activation, basal membrane degradation, leukocyte invasion, proliferation of vascular cells, neointima formation, changes of the extracellular matrix and cytokine participation (Cai and Schaper 2008). New capillaries form by sprouting or by splitting of the pre-existing vessels. Sprouting angiogenesis forms entirely new vessels differently from splitting angiogenesis. In splitting angiogenesis the capillary vessel extends into neighbouring vessel splitting it in two. Splitting angiogenesis is reorganization of the existing structures. It is also called intussusception (Folkman 1995; Risau 1997).

The ideal agent for therapeutic angiogenesis should have a potent effect and it should be spesific for the target organ or tissue, with sustained clinical benefit. Administration should be feasible and non-invasive (Emanueli and Madeddu 2006). It should maintain a high local concentration and adequate exposure time. Although some drugs show pro-angiogenic effect (Schaper 2005), so far only biological agents have been used to provide angiogenic therapy. There are three major ways to promote angiogenesis: protein therapy, gene therapy and cellular therapy (Al Sabti 2007).

Gene therapy for coronary disease

The argument supporting gene therapy as a method for therapeutic angiogenesis holds that gene therapy can overcome the short half-life of the angiogenic proteins by generating a prolonged local protein expression (Lopez and others 1998). Stimulation of vessel growth in heart by gene therapy has been under preclinical and early phase I-II clinical investigations over a decade (Edelstein and others 2004). Research studies have identified various angiogenic growth factors that can enhance new blood vessel formation. Studies in animal models have shown great potential of angiogenic gene therapy to treat myocardial ischemia. The results of clinical trials with gene therapy to enhance growth factor production have been disappointing, showing only mild improvements. To date, therapeutic angiogenesis remains at an early stage of development (Ahn and others 2008).

Vectors for gene therapy

Gene transfer can be can be performed by non-viral systems and recombinant viral techniques. The non-viral systems include plasmids and oligonucleotides and their derivatives. A growing number of vectors have been developed and are available for experimental and clinical gene transfer experiments (Dulak and others 2006). A substantial portion of the gene therapy protocols has been based on plasmids or short-strand nucleid acids, which are delivered through cell membrane in naked form or with the help of various chemical or physical methods. Because of the possible negative effect to a cell from the foreign nucleotide sequence, it is logic to assume that one of the main tasks of the cell membrane is to protect the cell from such invasion. The efficacy of the naked DNA transfection and the resulting gene expression is low and transient, lasting only for 1-2 weeks (Yla-Herttuala and Alitalo 2003). However, plasmids avoid many of the biosafety concerns associated with viral techniques. Plasmids are also easy to produce (Verma and Weitzman 2005).

The adenoviral DNA vector is a plasmid DNA that contains a portion of the viral genome. Recombinat adenoviral vectors contain several advantages for cardiac gene transfer. Since adenoviral replication depends on certain region of the viral genome, all recombinant adenoviral vectors have this region of its genome deleted, and are replication-deficient. These vectors are capable of infecting a cell only once, no viral propagation occurs, and the infected cell does not die as a result of vector infection. Viral entrance through cell membrane occurs via receptors, and the formed endosome release viral capsid. Nuclear entry of the viral DNA is completed upon capsid dissociation, and the viral DNA does not integrate into the host genome but remains in an episomal state. These viruses can be prepared in extremely high titers and they are able to infect both replicating and non-replicating cells. The production is relatively simple. Adenoviruses have shown to confer efficient transduction of cardiomyocytes after direct injection or perfusion approaches (Svensson and others 1999). Human recombinant adenoviral vectors have been the most used viral vectors in pre-clinical gene therapy models as well as in clinical gene therapy studies. The main disadvantage of the adenoviral gene therapy is the capacity to evoke intense immune and inflammatory reaction in the host (Edelstein and others 2004). In animal models, adenoviral vectors have been reported to cause myocardial and vascular inflammation, endothelial cell dysfunction, vasoproliferation and intravascular thrombus

formation. The death of a young human subject in a clinical trial conducted by adenoviral gene transfer has aroused the appreciation of the possibility of serious adverse events (Isner and others 2001). The other disadvantages of the adenoviral vectors include transient gene expression in animal models the cardiac gene transfer by adenovirus has generated a high-level gene expression of approximately one week. The duration of the transgene expression may improve in systems using cardiac specific promoters. Additional constraint for adenoviral use is the possible pre-existing neutralizing antibodies of the host and possible de novo development of antibodies to inhibit the re-administration of the same serotype.

Recombinant adeno-associated viruses (AAV) are derived from non-pathogenic parvoviruses. AAVs require cells to be doubly infected by a helper virus to replicate in nature. There are 11 serotypes of AAVs. Many of these serotypes have been shown to be effective in gene transfer (Chen and others 2005; Denby, Nicklin, Baker 2005; Nicklin and others 2001). The advantages of the AAV include the apparent lack of cellular immune response, the low immunogenicity depend on neutralizing antibodies. In comparison with adenoviral gene transfer, cardiac injection of recombinant AAV vectors produces less initial, but more sustained transgene expression. AAV can infect non-dividing as well as dividing cells and has the ability to stably integrate into the host cell genome (Gaffney and others 2007). There is a specific site in the human 19th chromosome, where most of the AAVs DNA integration takes place (Young and others 2000). This is an advantage compared to the retroviruses, which present a random insertion and thus the possibility of mutagenesis. Whether the expression level and the efficiency produced by AAV in cardiac diseases will be sufficient, remains to be seen. AAVs have spesific tropisms, altering of which might allow efficient targeted vector (Bartlett and others 1999).

Lentiviruses are derived from the family of retroviruses (Totsugawa and others 2002). The infection of cells by retrovirus is mediated by attachment of the viral envelope glycoprotein to the target cell-surface receptors. The RNA genome is converted to DNA by reverse transcriptase and eventually integrated to host genome (Sakoda and others 1999). This integration results in stable, prolonged, and potentially high expression of the therapeutic transgene, depending on integration site. The most characterized lentiviral vector system is based on the human immunodeficiency virus type 1 (HIV-1) (Rebolledo and others 1998). Only low titres are achievable at present and safety concerns regarding random insertion of reverse-transscripted DNA into the host genome are current drawbacks. Lentiviruses are able to transduce also non-dividing cells (Frimpong and Spector 2000).

Angiogenic receptors and factors

The VEGF- family consists of various members of the VEGF family having overlapping abilities to interact with a set of cell-surface receptors, which trigger responses to these factors (Yancopoulos and others 2000). The main receptors that seem to be involved in initiating signal transduction cascades in response to the VEGFs, comprise a family of closely related receptor tyrosine kinases consisting of three members now termed VEGFR-1, VEGFR-2 and VEGFR-3. In addition, there are a number of accessory receptors such as the neuropilins, which seem to be involved primarily in modulating binding to the main receptors (Soker and others 1996; Soker and others 1998). Mice lacking VEGFR-1 seem to have excess formation of endothelial cells which abnormally coalesce into disorganized tubules (Fong and others 1995), but

when the animals were engineered to express VEGFR-1 lacking its kinase domain appeared rather normal (Hiratsuka and others 1998). These data suggested a regulator role of decoy or ligand-binding supressor of VEGFR-1. Partial blockage of VEGFR-2 during development of VEGFR-1 resulted in less pathologic vasculature (Roberts and others 2004). There is also evidence of VEGFR-1 mediated pathological angiogenesis and inflammation (Carmeliet and others 2001). Furthermore, there is evidence of VEGFR-1 mediated suppression of proapoptocic gene expression (Li and others 2008). VEGFR-2 seems to mediate the major growth and permeability actions of VEGF. Mice engineered to lack VEGFR-2 fail to develop a vasculature and have very few endothelial cells (Carmeliet and others 1996). VEGFR-1 shows at least a 10-fold higher affinity to VEGF of only the extracellular domain, but about a 10-fold lower kinase activity than VEGFR-2 (Hiratsuka and others 2001; Sawano and others 1996; Sawano and others 1997; Shibuya 1995). On the other hand, VEGFR-3 is responsible for the development of lymphatic vessels (Dumont and others 1998a; Iljin and others 2001; Taipale and others 1999). Mice, which lacked a functional VEGFR-3 gene showed defective blood vessel development in early stage mouse embryos (Dumont and others 1998b). Thus, the signalling through VEGFR-3 has an essential role not only for lymphatic vessel formation but also for angiogenesis. Neuropilin receptors 1 and 2 regulate neuronal guidance and they bind to VEGF in an isoform spesific manner (Fujisawa and others 1997; Fujisawa and Kitsukawa 1998). In the endothelial cells neuropilins function as a supplementary receptors, regulating and enhancing the signalling of the VEGFs (Gluzman-Poltorak and others 2000; Karkkainen and others 2001).

Most of the research on the VEGF family so far, especially with respect to the angiogenesis, has focused on VEGF-A, which has different isoforms (Yla-Herttuala and Alitalo 2003). VEGF-B binds to both VEGFR-1 and neuropilin-1. VEGF-B is implicated in angiogenesis by its role in the regulation of extracellular matrix degradation, cell adhesion and migration of endothelial cells (Olofsson and others 1998). VEGF-C is a ligand for both VEGFR-2 and VEGFR-3 (Joukov and others 1996). VEGF-C is synthesized as a prepropeptide and subsequently undergoes proteolytic maturation. Only the fully processed form is able to activate VEGFR-2 (Joukov and others 1997). VEGF-D is structurally very similar to VEGF-C and it also binds to VEGFR-2 and VEGFR-3 (Achen and others 1998). VEGF-D is mitogenic for endothelial cells and thus may play a role in endothelial cell regulation. The expression of VEGF-D is prominent in heart and skeletal muscle. VEGF-E is the collective term for a group of proteins with homology to VEGF-A that are encoded by certain strains of the orf parapoxvirus (Robinson and Stringer 2001). It possesses about 25% amino acid identity to mammalian VEGF (Lyttle and others 1994).

Fibroblast growth factors (FGFs), have profound effects in various endothelial cell assays, but are also known to be nonspecific in that they could act on many other cell types (Colvin and others 2001; Ellman and others 2008; Kapur and Rade 2008; Ornitz and Itoh 2001). The FGF family is a large group of proteins, which share 30–70 % identical primary sequences. Of these, FGF-1, FGF-2, FGF-4 and FGF-5 have been used for angiogenesis studies (Javerzat, Auguste, Bikfalvi 2002). HGF is also a potent mitogen of endothelial cells and it also activates many other types of cells as well and has thus cardioprotective properties (Ono and others 1997). In addition, many other growth factors that are not vascular endothelium-specific are also required for blood vessel formation, such as members of the platelet-derived growth, although these factors also have critical roles for many other systems as well (Zhang and others 2008).

Administration strategy for Angiogenic Therapy

Local gene delivery to the target organ should be prefered, when optimal treatment-effect versus side-effect profile is aimed (Simons and others 2000). Percutaneous delivery methods are readily available due to the wide spread catheterization techniques in cardiology. The gene material can be injected directly into coronary circulation under angiographic inspection. Direct intramyocardial injection technique is possible via percutaneous route if special catheters are passed through aortic outflow track into LV (Kastrup and others 2005; Rutanen and others 2004a). Venous cathetrization enables the use of coronary sinus. With a special side needle the therapy can be injected through veins into the myocardium (Siminiak and others 2006). Gene therapy can also be delivered from stents using cellular basesd techniques (Koren and others 2006). During surgery as a concomitant procedure, the injection can be easily performed, but as a solitary procedure the injection itself causes risks and burden to the patient.

Side effects of the angiogenic treatment

The disadvantages of gene therapy are dependent in the vector used, gene transducted and the route of the delivery of the treatment. In any case, introduction of foreign genetic material can be considered as a potential risk. The exposure to the vector, especially when viral vectors are used might possess risks due to inflammatory response (Isner and others 2001). Inability to regulate the gene expression might possess risk, at least in case of overdose in targeted (Lee and others 2000b; Schwarz and others 2000; Springer and others 1998) or non-targeted tissues, especially in case of malignancy (Folkman 1971), or retinopathy (Aiello and others 1994; Miller and others 1994). Some angiogenic factors have also shown to accelerate atherosclerosis (Celletti and others 2001; Inoue and others 1998) and cause odema (Dvorak and others 1999; Senger and others 1983). The uncontrolled insertion of the viral-originated DNA of retroviruses to the host genome might cause carcinogenic effect (Duesberg 1987).

Human trials for therapeutic angiogenesis in coronary disease

In a trial, 6 patients were receiving naked plasmid VEGF-C by transmyocardial injections via percutaneus catheter. The VEGF-C transfected patients experienced reduced angina, reduced nitroglycerin consumption and reduced ischemia by electromechanical mapping as well as myocardial perfusion by SPECT-sestamibi scanning for up to 90 days after GTx when compared with images obtained after control procedure (Vale and others 2001)

In a non-randomized phase I multi-center trial 30 patients Class III or IV angina refractory to medical therapy were enrolled to receive intramyocardial injections of VEGF-C in a dose-escalating fashion. The procedure was performed via a mini-thoracotomy. Patients were followed for clinical events after one year by hospital records, follow-up visits or telephone contact. At the most recent follow-up, no patients had Class IV angina, and only three (11.5%) had Class II angina, while the remaining had Class I or II angina. Compared to baseline, average angina class decreased from 3.6 ± 0.5 to 1.3 ± 1.0 after the first year. This benefit persisted to 24 months when the mean angina class was 1.5 ± 1.2 ($p < 0.05$). Major clinical events such

as death, myocardial infarction (MI, or heart attack) and repeat revascularization were uncommon during the first year but more frequent after one year at a rate consistent with the severity of the underlying disease in a population with advanced atherosclerosis. The majority of events were the result of progression of disease in areas of the heart remote from the site of injection (Reilly and others 2005).

In VIVA trial, a total of 178 patients with coronary artery disease were randomized to receive high or low dose of recombinant VEGF-A165 protein or placebo. The protein or treatment was infused intracoronary route. The primary end point was treadmill exercise capacity at 4 months. There was no significant difference between the groups, anyhow the high dose VEGF-A improved significantly in angina class as compared to placebo (Henry and others 2003).

The KAT (Kuopio Angioplasty phase II) Trial investigated the efficacy of intracoronary plasmid/liposome VEGF-A165, adenoviral VEGF-A165 or placebo injection in conjunction with coronary stenting procedure. Primary end point included restenosis and myocardial perfusion at 6 months. Restenosis was similar between the groups, but adenoviral VEGF-A165 group showed improved perfusion at the end of the study (Hedman and others 2003).

In a study conducted by Losordo et al., 19 patients were randomized to percutaneous intramyocardial catheter delivered naked VEGF-C plasmid in three different doses or placebo. Patients receiving VEGF-C plasmid showed significant reduction in angina class at 12 months (Losordo and others 2002).

In the EUROINJECT One-study, a total of 80 patients were recruited to receive either naked plasmid VEGF-A165 or placebo by percutaneus intramyocardial injections as a sole therapy. At 3 months, there was no difference between the groups assessed by the size of the perfusion defect or CCS class. However, catheter based mapping of the left ventricular motion at the injection site was better in the treatment group, when compared to the placebo group (Kastrup and others 2005).

In the REVASC (Randomized Evaluation of VEGF for Angiogenesis in Severe Coronary Disease) trial, 67 patients were randomised to either maximal medical therapy or to receive adenoviral VEGF-A121 with intramyocardial injections by open thoracotomy. At 3 months after randomization, there was no difference between the groups, but at 6 months in the adenoviral VEGF-A121 group exercise test was significantly improved (Stewart and others 2006).

In the GENASIS trial a total of 295 patients, who were not suitable for traditional revascularization procedures were enrolled to receive naked plasmid VEGF-C or placebo. The treatment was given by intramyocardial injections with a percutaneous catheter. The primary efficacy endpoint in the GENASIS clinical trial was an improvement of at least one minute in exercise tolerance time from baseline to three months. The primary endpoint showed no statistical difference between the groups. The data indicated considerable overlap in results between the treatment and placebo groups for the secondary endpoints as well, and no clear dose effect was seen.

4. Aims of the present study

The main purpose of this study was to assess cell proliferation methods in treating ischeamic heart disease in porcine model by using MRI, PET and SPECT.

Specific Aims:

1. Testing the angiogenic potency of ad-VEGF-C administered intramyocardially (I)

2. Improving the Ameroid occlusion in model of myocardial ischaemia by time-adjusted ligation of the stenosed vessel (II)

3. Assessing spontaneous regeneration in ischeamic-infarcted porcine heart in terms of cellular proliferation, perfusion and angiogenesis (II-III)

4. Assessing the efficacy of autologous myoblast transplantation in ischemic/infarcted porcine heart (IV)

5. Materials and Methods

Agreements and notifications

The Local Ethics Committee of Helsinki University Central Hospital and the Provincial State Office of Southern Finland approved all animal experiments and notification was submitted to the Board for Gene Technology.

Treatment and anaesthesia of the animals

Landrace piglets were used as laboratory animals to provide a model of coronary heart disease. Fifteen animals (weight 17-27kg) was used in study I, 11 animals (weight 18-26kg) in study II-III and 44 animals (weight 18-26kg) in study IV. All animals were maintained and treated in accordance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publications No. 86-23, revised 1985). General anaesthesia was induced in the animals in a fasting state by intramuscular azaperone (4-8 mg/kg), ketamine hydrochloride (5 mg/kg) and atropine sulfate (0.05mg/kg). They were intubated and ventilated with Siemens Servo ventilator 900C (Siemens Elema AB, Solna, Sweden). Anaesthesia was maintained with additional boluses of intravenous ketamine hydrochloride (50-100 mg) and continuous infusion of propofole (4-12mg/kg/h). In the case of operative procedures, pancurone (0.1 mg/kg) was used for muscle relaxation and fentanyl ($0–5$ μg/kg) for pain management. In that case, prophylactic intramuscular ceftriaxon 500 mg and intravenous ranitidine 25 mg were given. Postoperative pain was treated with intramuscular diclophenac acid (25-37.5mg).

The study implementation

Ameroid model of myocardial ischaemia (Study I-IV)

A left thoracotomy was performed anterolaterally at the level of 4th intercostal space. Pericardium was incised and the left auricle was displaced. Epicardium was incised over the proximal left circumflex artery (LCx) just after the bifurcation of the left main coronary artery. If the animal exhibited trifurcation of the left main coronary artery i.e. a significant left intermediate coronary branch was apparent, the animal was discarded from the study. The proximal LCx artery was dissected free and an Ameroid constrictor with an internal diameter of 2.5 mm (manufacturer: Research Instruments SW, Escondido, CA, USA) was placed around the LCx. The wound was closed with synchronous lung expansion to minimize the amount of pneumothorax and the animal was allowed to recover.

Administration of adVEGF-C and adLacZ vectors (Study I)

At three weeks after the Ameroid placement, re-thoracotomy was performed at the 5th intercostal space. The lateral wall of the LV was exposed. A partial resection of the fifth rib was performed, if needed to receive adequate exposure. Adenoviruses encoding VEGF-C (adVEGF-C) or control beta-galactosidase (adLacZ) were injected directly into the myocardium as ten separate 0.2 ml doses in the lateral wall of LV between LAD and LCx with 2x1011 viral particles (vp) in a total volume of 2.0 ml diluted in saline with a 2.0 ml syringe and a 0.2 ml Microflex infusion set (Laboratoires Pharmaceuticals Vygon, Ecouen, France). Prolene® purse-string suture was placed to encircle the area of the injections. The wound was then closed, and the animal was allowed to recover.

Ameroid model with subsequent coronary ligation (Study II-IV)

After the pericardium was opened, and the Ameroid constrictor was positioned around the proximal LCx artery, a loose non-absorbable 3-0 band was applied around LCx artery. The thread loupe was placed just distally to the Ameroid constrictor, and the thread ends were conducted via the third and fourth intercostal space and embedded in the muscular layer inside a 2 cm long polyethene tube, which was cut from a needle holder. The wound was closed in three layers and the animal was allowed to recover. Two weeks (study IV) or three weeks (study II and III) later, another operation was performed. The plactic tube and the threads were exposed by dissecting through a separate skin incision. The loose band around LCx vessel was tightened, by tying the threads firmly around the 4th costa, and the wound was closed. The animal was observed for one hour for possible arrhythmias and in the case of ventricular fibrillation, electrical cardioversion was executed and if sinus rhythm was not reached, intravenous amiodarone 5mg/kg was administered during re-cardioversions.

Myoblast harvest and transplantation (Study IV)

Two weeks after the Ameroid placement at the time of ligation procedure, skin was incised at the lateral thigh. A biopsy of 1cm3 was harvested from the lateral vastus muscle. The wound was sutured in layers. Muscle biopsy was eventually processed in the laboratory as described below. Two weeks later, after the pre-treatment cardiac assesment under general anaesthesia a thoracotomy was performed in the previous wound. The lateral free wall of the LV was exposed and 2 x 106 myoblasts diluted in 2 ml of Dulbecco's modified Eagle medium (DMEM; Gibco-BRL Company, Grand Island, USA) were injected in the lateral wall of the LV. Injections were 0,2ml each applied over the free lateral wall of LV. Area of injections was encircled by a prolene® suture.

Animal euthanasia and histological sampling (Study I-IV)

At the end of each study, animals were anaesthetized and euthanized with an overdose of potassium chloride. The tissue samples were collected from each heart. The animals in which the area of the treatment was marked with prolene® suture, the samples were collected from the marked area. In other animals a representative tissue sample of the LV lateral wall was

harvested. Another sample at the penumbra of the treatment area or representative area of the lateral wall was collected. Control samples were collected from each animal from the antero-lateral septum of the LV.

Preparation of the viral constructs and myoblast processing

Recombinant adenoviral vectors (Study I)

Adenoviral constructs encoding the complete human prepro-VEGF-C or nucleus-targeted LacZ were provided by Juha Rutanen (AIV-Institute, University of Kuopio). The constructs were prepared as described: "Cytomegalovirus (CMV) promoter was excised from the pcDNA3.1 vector (Invitrogen), and a full-length human VEGF-C cDNA containing the 1997-bp sequence was excised from the previously constructed VEGF-C pREP7 expression vector. A human growth hormone polyadenylation signal was excised from an -MHC gene promoter construct (a gift from Dr Jeffrey Robbins). The CMV promoter, VEGF-C cDNA, and the polyadenylation signal fragments were ligated into a pCRII vector (Invitrogen). The transcriptional unit was cloned into pAdenogal vector. This construct was then used to generate recombinant adenovirus. VEGF-A (murine VEGF164) and nucleus-targeted lacZ adenoviruses were constructed in a way similar to that previously described. Replication-deficient E1-E3–deleted clinical GMP–grade adenoviruses were produced in 293T cells. Adenoviruses were analyzed to be free from helper viruses, lipopolysaccharide, and bacteriological contaminants." (Hiltunen and others 2000)

Myoblast processing (Study IV)

Skeletal myoblasts were obtained from porcine vastus lateral muscle. Under sterile conditions, a 1cm3 muscle sample was cut, and washed twice in ice-cold $Ca2+$-$Mg2+$ -free Hank's balanced salt solution (Gibco, Carlsbad, CA) containing antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). It was then carefully minced, and the fragments were enzymatically dissociated by several incubations in serum-free DMEM, containing 0.25% trypsin and 1mg/ml type IV collagenase (Worthington, Lakewood, NJ). Between incubations, the fragments were further dissociated by vigorous pipetting. Cells were then filtered from the digested tissue, were centrifuged 400 g for 10 minutes and washed twice in DMEM containing 10% fetal calf serum and antibiotics. The resulting cell suspension was plated for 45 minutes in uncoated plastic 25 cm2 cell culture flasks to remove fibroblasts by the differential attachment technique. Unattached cells were then replated on cell culture flasks coated with gelatin (Sigma, St Louis, MI). Cultures were incubated at 37oC in a humidified atmosphere of CO2 (5%) and air (95%). After 1 week in culture the cells were detached with trypsin and subcultured in 1:3–ratio. Three to four days after subculturing, the cells were collected for transplantation and staining. For transplantation the cell-count was optimized to 1x106 cells/ml in serum-free DMEM.

Examination methods of the injured heart

Angiography (Study I-IV)

Coronary angiography was performed in vivo during anaesthesia just before the treatment (study I and IV) and four weeks thereafter. In the studies of spontaneous myocardial recovery from Ameroid induced stenosis and subsequent ligation (study II and III), angiography was performed one and four weeks after the ligation. Siemens multistar T.O.P. angiography equipment (Siemens AG, Munich, Germany) was used with Hexabrix 200 mgl/ml contrast agent (Laboratoire Guerbet, Roissy, France). Two observers blinded to treatment groups visually determined the LCx size in a semiquantitative manner (1= small, 2=normal, 3=large) and estimated the percentage stenosis of the LCx.

Schwartz classification was used to grade the collateral vessel. In this classification, the filling of the stenosed vessel determines the function of the collaterals (Schwartz and others 1984). The classification is divided in four grades (0-3). Configuration of collaterals were divided into three areas 1) antegrade collaterals locally around the Ameroid region, 2) the treatment area, which was the location of injections, 3) peripheral collaterals. The treatment area consisted of collaterals from the first branch of LAD to the first branch of LCx or peripheral LCx and from the second branch of LAD to the first branch of LCx or peripheral LCx branches. The 'treatment area index' was calculated by change in number of visible collaterals between 3-week and 7-week studies in the treatment area divided by the change in collaterals in the entire left coronary area (study I). The Schwartz gradus and treatment area index were compared between groups.

SPECT (Study I and II)

Technetium 99-m sestamibi SPECT was performed under general anaesthesia. Perfusion was assessed with the Toshiba GCA-7200A/UI dual head gamma detector (Toshiba Corp, Tokyo, Japan) with low energy high-resolution parallel hole collimators (NDCL-701A), 64x64 matrix, and a 20% window. Image acquisition was performed with 180-degree rotation and 30 stops at 20 seconds/stop. Electrocardiogram (ECG) was used to guide the acquisition, 16 frames between each R-R segment were obtained. Rest and stress imaging were performed for each animal with a minimum time interval of 4 hours between the tests. Rest imaging was assessed at a dose of 0.3 mCi/kg of 99mTc. Pharmacological stress was induced with a dobutamine intravenous infusion of 40 μg/kg/min, the dose increasing every three minutes by 40 μg/kg/min up to 12 minutes, and an intravenous bolus of 0.3 mCi/kg 99mTc was given at 11 minutes.

The SPECT images were processed with GCA-7200A/UI software (Toshiba). Multislice diagrams were automatically reconstructed from short axis slices. These diagrams were divided into 48 segments. The segment, which obtained most counts was assigned as a value of 100. Values of other segments were normalized to this segment. Global LVEF, regional wall motions, and cardiac volumes were supplied by the software. Left ventricular motion and thickening were acquired under stress separately, and presented as multislice diagrams on a ten-colour scale. LV was divided into 13 segments in the motion and thickening multislice diagrams, of

which the four posterolateral segments represented the LCx perfusion area. The mean motion or thickening in all the other segments was used as a control area measure.

Image interpretation: Ejection fractions were calculated as the difference between end-diastolic and end-systolic volume divided by the end-diastolic volume. Improvement in LVEF under stress was presented as percentage difference between rest and stress end-systolic volumes. 90 degrees of circumferential count centred on the segment of maximal reversible ischaemia in a multislice diagram was determined to represent the LCx perfusion area in each image. Segmental counts in the whole LCx area were summed to reach a value representing both depth and extent of ischaemia. Apical segments were eliminated due to naturally low counts. Difference in counts between stress count sum and rest count sum at the LCx area presented the amount of reversible ischaemia.

PET (Study I and III)

Positron emission coincidence detection (CoDe) imaging was performed using dual-head coincidence detecting gamma camera (Millenium VG-CoDe 5, GE Medical Systems, Milwaukee, WI, USA). This technology is based on a combined gamma camera and computed tomography, allowing all data gathering for attenuation in one imaging session (Bocher and others 2000). Fasting animals were anesthetised as described above. 25 g intravenous glucose and 6 IU of insulin were given to induce an insulin clamp. After 45 minutes, 3-4 mCi of 18-fluorodeoxy-fluoroglucose (18F-FDG) was administered intravenously 50 minutes prior to imaging. Images were acquired and multislice diagrams were reconstructed from attenuation corrected short axis slices.

Analysis of the images was performed by a single observer blinded to randomisation. For each animal, the SPECT images were aligned for direct comparison. The level of 18F-FDG uptake at three weeks imaging in the LCx area was determined. In the study I, the change in 18F-FDG uptake between 3- and 5-week images and between 5-and 7- week images in the LCx was determined as improvement or deterioration in collateral function. Results between any two time-points were graded in a semiquantitative manner: improvement = 0, no change = 1, deterioration = 2. Acquired grades were compared between groups. In the study III, 90 degrees of circumferential count centred on the posterolateral portion of the LV was assigned as the lesion area. The mean count at the lesion area was compared to the mean count of the rest 270 degrees of the multislice diagram.

Magnetic resonance imaging (Study III and IV)

Magnetic resonance imaging was performed with a 1.5 T Siemens Sonata scanner and a body array coil (Siemens AG, Erlangen, Germany). The animals were anestehetized and positioned supine. Long axis of the LV was imaged in two perpendicular planes. Short axis images were acquired with the three following MR sequences. Firstly, LV volume measurements and regional wall thickening analysis were obtained by fast imaging with steady precession (True-FISP) cine images. The sequence parameters were: Slice thickness 7mm, short axis sections 7mm apart, temporal resolution 33 ms, 300 cm, matrix 256 pixels, 1.57 ms, 5.4ms flip angle 60° (Francois and others 2004). Secondly, to assess myocardial perfusion, an inversion recovery

turboflash image series were obtained during dipyridamole stress (Persantin®0.5 mmol/kg in four minutes) and gadolinium- diethylenetriamine penta-acetic acid bolus injection (Magnevist®, 0,10 mmol/kg). The four short axis planes were imaged repeatedly at every second for three minutes to observe the myocardial first pass of the contrast agent. The sequence parameters were: Slice thickness 8 mm, 300 cm, matrix 256 pixels, 0.99 ms, 183 ms, flip angle 8° (Sakuma and others 2005).

Thirdly, the measure of infarct and viability, ten minutes after contrast injection, all long and short axis planes were imaged with an inversion recovery late enhancement sequence. The sequence parameters were: slice thickness 7mm, field of view 300 cm, matrix 256 pixels, inversion time 270 ms, time to echo 4.3 ms, time to repeat 750ms (Selvanayagam and others 2005).

Image analysis was performed with Impax Client software (Release 4.5, Agfa-Gevaert Group NV, Ridgefield Park, NJ) in study III and IV, and Argus software (Siemens AG with Argus, Erlangen, Germany) in study IV. Left ventricular global and regional parameters were assessed from images obtained at one and five weeks after ligation. Global parameters, LVEF and muscle mass, were assessed from short axis cine image series. The endocardial border was drawn at diastole and systole at each section. The LV cavity area was multiplied with the section distance, and the global volumes were calculated as a sum of all short axis sections at end diastole and systole as end-diastolic volume (EDV) and end-systolic volume (ESV). EF was calculated as (EDV-ESV)/EDV. To obtain muscle area the epicardial border of each section was drawn.

Wall thickening and perfusion of the ischemic LCx region at the free LV wall was compared with the normal left anterior descendens (LAD) region at the anteroseptal wall on the short axis sections. Wall thickness at LCx region was measured at systole and diastole. The section with the smallest fractional thickening was selected to represent the ischemic area. The fractional thickening of LCx region was normalized to that of LAD region in the same section. Perfusion of the same LCx and LAD regions was observed from the contrast agent transit into the myocardium during the first pass imaging. Perfusion was quantified by a slope of pixel intensity increase versus time. The LCx slope was normalized to LAD. The amount of infarcted myocardium was quantified from late enhancement images by dividing the LV into the 17 segments according to AHA statement (Cerqueira and others 2002). Each segment was graded according to enhancement: 0 = no scar, 1 = subendocardial scar, 2 = transmural scar. Relative amount of scar tissue was calculated by dividing summed scar units by total myocardial units of 34 (17 times 2 units).

Short axis cine images were assessed for parameters of diastolic function. LV cavity time-volume curves were acquired from data points of TrueFISP cine images. LV volume was measured throughout the cardiac circle. Peak filling rate (PFR) was the maximum rate of filling (ml/s) between two frames in early diastole. Time to peak filling rate (TPR) was the time (ms) from the end systolic frame to the time point of PFR. Duration of diastole (DD) was the time (ms) of the cardiac filling, and peak ejection rate (PER) was the maximum rate of left ventricular emptying between two frames during systole (ml/s). Early diastolic filling was the volume change of LV during the first third of diastole.

Histological assessment (Study I-IV)

After above described studies the animals were euthanized and autopsied, the heart was removed and the size and location of the infarction was macroscopically evaluated. In addition, tissue samples were collected for histological evaluation from the lesion area, from the border area and from the remote area of the myocardium. Hematoxylin-eosin staining was performed for asssesment of infarction and inflammation.

In order to detect the microvessels, immunohistochemistry was performed with von Willebrandt factor antibodies (Rabbit Antihuman vWF, Code No A0082, Dako A/S, Glostrup, Denmark) and Vectastain® Elite ABC kit (Vector Laboratories inc, Burlingame, CA, USA) from adjacent 5-μm sections (studies I-IV). These samples were analysed under a light microscope (Olympus Optical) by a single observer blinded to randomization. Microvessels were counted in three randomly selected high- power fields in the treatment area and from apical control area. The sample in each animal with the highest vessel density in the treatment area was considered to represent maximal microvessel development (study I-IV). Avidin-biotin-horseradish peroxidase with diaminobenzidine (DAB; Vector Laboratories inc, Burlingame, CA, USA) was used for immunohistochemistry of vascular smooth muscle (study I). Blood vessels were immunostained with a mouse monoclonal antibody against α-smooth-muscle antigen (Clone 1A4, 1:250 Sigma, St Louis, Missouri, USA)

The antibodies were polyclonal rabbit-anti-cyclooxygenase-2 (LabVision Corp., Fremont, CA, USA), monoclonal mouse-anti-desmin (Dako A/S, Klostrup, Denmark), monoclonal rabbit-anti-Ki-67 (LabVision) and monoclonal mouse-anti-human macrophages (cross-reactive with pig macrophages, Serotech Ltd., Oxford, UK). With these, Ventana red alkaline phosphatase fast red detection kit was used (study IV). For improved localization of proliferating nuclei, the sections were first stained for cardiac troponin-T using the Ab-1 monoclonal antibody (Labvision, Fremont, CA, USA) and detection after incubation with alkaline phosphatase-conjugated secondary antibody (study IV). Thereafter the sections were double stained for Ki-67 using monoclonal rabbit-anti-Ki-67 (study IV). The number of dividing cells and dividing myocytes from adjacent sections stained for Ki-67, and troponin-T were counted by an experienced pathologist blinded to the samples. The area of the sample on each slide was measured from with the PhotoShop software (Adobe Systems Inc., San Jose, CA, USA) after scanning. The density of the proliferating cells was recorded as the number of Ki-67-positive cells divided by the sample area (cm2). Percentages of Ki-67-stained myocytes in the lesion area, in the border area and in the remote area were assessed. The total proliferation index was defined as the proportional amount of Ki-67-positive cells in the lesion area, in the border area and in the remote area.

During the myoblast cultivation, the cell lineage was evaluated by the expression of desmin (study IV). The cells were fixed in –20°C methanol, washed with PBS, and incubated with monoclonal anti-desmin antibody for 1 hour. They were then washed with PBS and incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (Dako, Glostrup, Denmark). Immunoreactivity was visualized using the 3-amino-9-ethyl-carbazole (AEC) chromogen. The cells stained 80-90% positive for desmin.

For localization of skeletal myoblasts post mortem, skeletal muscle specific anti-nebulin immunohistochemistry was used. Collagen was stained with Van Gieson's stain. The samples were analyzed under a light microscope (Olympus Optical) by an experienced pathologist blinded to randomization. Photographs of histologic sections were taken with an Olympus

AX70 microscope (Olympus Optical) and analySIS software (Soft Imaging System). The im-munostained sections were photographed in five random spots and the pixel intensity values were limited to 8 bits per sampled pixel (range of values, 0-255). The cut off point for staining was set on a pixel value of 60/255 shades of gray, and the stained pixel fraction was counted using imageJ software (National Institutes of Health, USA).

Other examination methods

Imaging of retina

Adverse retinal neoangiogenesis was assessed (study I). Dilatation of the pupils was carried out with 0.5 % tropicamide, and 10% phenylephrine. Central retinal area around the papilla was photographed by a Genesis Hand-Held Fundus Camera Camera (Kowa Europe, GmbH, Dus-seldorf, Germany) and Kodakrome Elite 100 ASA film (Eastman Kodak company, Rochester, NY, USA). A total of six eyes of three animals in the adVEGF-C group, and four eyes of two animals in the adLacZ group were photographed. Interpretation of retinal neovascularization was executed by an experienced ophthalmologist blinded to treatment groups.

Statistical methods

Data were analyzed in a blinded fashion. Paired t-test was used to test the significance of dif-ference between two time points in within one group, when Gaussian distribution was assumed (study I-III). When non-Gausssian distribution was assumed, Wilcoson test was used (study IV). Different groups were compared with two-tailed non-paired t-test in case of Gaussian dis-tribution (study I-III) and with Mann-Whitney test in case of non-Gaussian distribution (study IV). Fisher's exat test was used with contingency tables (study IV). Data was presented as mean ± SEM (Study I) or as median and range (study II-IV). Differences between multiple factors were compared with Kruskall-Wallis test (study III). A p-value less than 0.05 was considered statistically significant
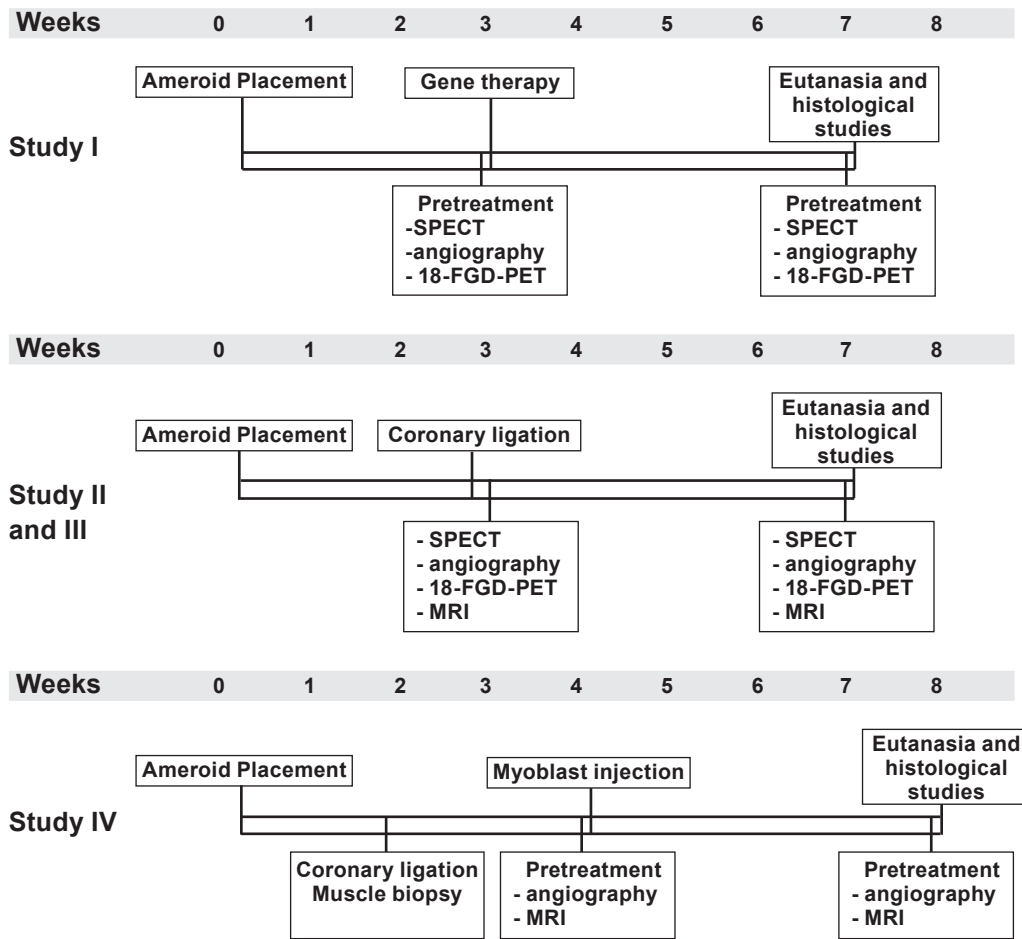
Figure 1. Study protocols. Study protocols. Ameroid is a slowly swelling ring applied around coronary artery, causing gradually increasing stenosis of a coronary artery. Angiogenic gene therapy was carried out with vascular endothelial growth factor-C (study I). Ligation of the narrowed coronary vessel was performed in a phase of nearly occluded artery, when already formed collateral vessels would protect the myocardium from excessive infarction (study II-IV). Autologous myoblasts were harvested from vastus muscle and after cultivation of 2 weeks the cells were injected directly to the myocardium (study IV). SPECT = single emission computed tomography, 18-FDG PET= 18-fluorodeoxyglucose positron emission tomography, MRI= magnetic resonance imaging.

6. Results

Study I

Overall assessment

Survival of the animals was 12 out of 15, and mortality was 20%. Median weight of the animals was 34 (range 27 to 42kg) at three weeks and 61 kg (range 50 to 73kg) at 7 weeks. One animal developed an infarct at the lateral LV wall, which comprised 6% of the LV mass according to SPECT studies. Retinal photograph was unable to detect any neovascularization in the examined retinas. Adverse effects of adVEGF-C or adLacZ were not found in any of the animals.

Progression of the LCx stenosis durig the study

Pretreatment angiography at three weeks after the Ameroid placement showed a median 75% stenosis in LCx, range 75 to100% in the adVEGF-C group. The LCx stenosis at 7 weeks, had progressed to median100%, range 90 to 100% (p=0.041, Wilcoxon test). In the adLacZ group, the stenosis at three weeks' was median 75%, range 60-75% and progressed to median100%, range 85-100% at seven weeks' (p=0.042, Wilcoxon test). The degree of LCx stenosis did not differ between groups at 3 weeks' or at the 7 weeks' angiography (p=0.15 and p=0.64, respectively, Mann-Whitney test).

Angiographic evidence of the collateral vessel formation

Significant collateral vessel formation was detected in both groups between 3-weeks' and 7-weeks' angiographies. In the adVEGF-C group, the median Schwartz grade at 7-weeks' angiography was median 2.75, range 2.0 to 3.0 and in the LacZ group, median 2.0, range 1.0 to 2.5 (p=0.052, Mann-Whitney U-test). The treatment area index, which measures the relative amount of collateral vessels at the injection site, demonstrated a significant accumulation of collaterals in the treatment area when the adVEGF-C group was compared to the LacZ group (p=0.004, Mann-Whitney U-test).

Evaluation of the 18-FGG PET

Change in myocardial metabolic substrate plays a key role, when ischaemic myocardium is judged by positron emission tomography. Ischemic myocardium favours the use of glucose over fatty acids, and 18F-FDG glucose analogue cellular uptake increases in a hypoperfused myocardium. Semiquantitative assessment of 18F-FDG uptake suggested improvement in myocar-

dial substrate change in the LCx region in the adVEGF-C group, when compared to the control group (p=0.052, Mann-Whitney U-test).

Measurement of myocardial perfusion by SPECT

Myocardial perfusion judged by 99Tc sestamibi SPECT detected no difference between the adVEGF-C and adLacZ groups (p=0.34, Mann-Whitney U-test). Global and local systolic wall thickening was measured by 99Tc sestamibi SPECT. Local systolic LV wall thickening in the LCx area decreased during the study period in the LacZ group (p=0.042), whereas the decrease at the same area in the VEGF-C group did not reach statistical difference (p=0.10). Similarly, global LV wall thickening in the LacZ group deteriorated significantly (p=0.043), while the ad-VEGF-C group preserved the pretreatment status (p=0.50) despite progressive LCx stenosis.

Decrease in LVEF during dobutamine stress compared to rest imaging is highly indicative of myocardial ischaemia. Decrease in stress EF to the corresponding rest EF was a median 9% (range –28-16%) in the adVEGF-C group, and 6% (range 4-17%) in the LacZ group. At the 7-week study, decrease was a median 8% (range –8-23%) in the adVEGF-C group and a median 13% (range 11-38%) in the adLacZ group. Difference between the groups at 7-weeks was insignificant (p=0.09). Deterioration in LVEF between 3 and 7 weeks was more evident in the LacZ group (p=0.043, Wilcoxon test), whereas the VEGF-C group suggests a similar course in LVEF (p=0.091, Wilcoxon test).

Histological evaluation of the microvessel count

We were unable to discover any adverse effects in either group in the treatment area, or other parts of the myocardium. Diffuse fibrosis and lymphocytic infiltration were found in both groups lateral wall at the area of ischaemia and injections. Myocardial samples at the treatment area in the adVEGF-C group showed more microvessels in the Von Willebrand factor staining compared to the adLacZ group (p=0.03, Mann-Whitney U-test). Apical control samples remote of the injections showed no difference in microvessel count between groups (p=0.15, Mann-Whitney U-test). Enlarged microvessels in αSMA immunostaining was found in the adVEGF-C group at the site of injections at 6 days after gene transfer, but not in the adLacZ group.

Study II-III

Overall assessment

Eight animals out of 11 survived the whole study period (mortality was 27%). Median weight of the animals was 63kg (range 59 to 76). Two animals died due to banding, and one animal died because of displacement of intubation tube.

LCx stenosis after the ligation procedure

Mortality in the ligation group was 3 animals out of 11 (27%). One week after banding of the Ameroid-stenosed LCx artery, angiography showed subtotally or completely occluded in all but one animal. In this animal the stenosis was estimated as 87% one week after the ligation, but second angiography revealed total occlusion four weeks later. Rentrop grade of the lateral wall collaterals was 2.7 ± 0.4 and 2.5 ± 0.5 at one and five weeks after banding, respectively (p=n.s.). When the stenosis after the ligation was compared to the angiography of 12 animals of the study I at three weeks, significantly more constant stenosis was shown (p=0.0009).

LV infarction measurement

Stress SPECT compared to the respective rest SPECT showed stress-induced perfusion defect at one week after the banding, which resolved until the final study. The perfusion defects were localized at the LCx area; other areas showed normal perfusion. Recovery of the perfusion at five weeks after banding was statistically significant comparing to the one week's studies (p=0.02, paired t-test).

In MRI, Myocardial scar was seen in five animals but not in three animals. There was 100% agreement in the detection of myocardial scar in individual animals between the PET, SPECT and MRI studies. Scar size assessed by MRI decreased significantly from one week (10.5% ± 7.9%) to five weeks (6.8% ± 5.3%, p=0.03, paired t-test).

PET images showed static irreversible perfusion defects in five animals. SPECT, MRI and PET had 100% agreement in the detection of scar. There was a considerable amount of individual variation in glucose uptake in the lesion area. The animals with a detectable scar on MRI had a decreased glucose uptake. Two of three animals, which had no detectable scars in MRI had an increase in the glucose uptake (as a sign of myocardial ischemia), and the third animal had near-normal glucose uptake in the LCx area at the one week study. The animals with abnormal glucose uptake at one week showed a shift towards a less deranged glucose uptake at five weeks and energy consumption improved significantly over time (p=0.03, paired t-test).

In histology all but one animal showed myocardial infarction in HE-staining, including the prematurely died individuals. Most common myocardial lesion area was transmural myocardial infarction at least 5 cm2 and other animals showed scattered subendocardial scar. Location was left ventricular lateral wall with some extension to the inferoposterior region. Interestingly, the two animals with largest transmural scars (up to 12 cm2) were those with biggest antegrade collaterals seen at the second angiography. In one animal with strongly right dominant coronary anatomy, only a small subendocardial scar was detected at the lateral wall. The animal

with non-occluded LCx at one week after the ligation showed no macroscopic scar but diffuse fibrosis in histology, indicating ischemia at the lateral wall.

Border area ischemia at the infarct penumbra

 Stress SPECT studies compared to the corresponding rest SPECT studies showed that a perfusion defect was induced at one week after banding, but that the defect had resolved by 5 weeks. The difference between these two study points was statistically significant (p=0.02, paired t-test). On MRI, the difference between signal intensity change rate in the LCx area and the control area was a median of 34° (range 19°-59°) at one week and 15° (range 0°-34°) at five weeks after banding (p=0.0065, paired t-test).

Systolic function assessed by MRI

At one week after banding, MRI showed a reduction in the systolic LV-wall thickening of the lateral wall compared to non-ischemic parts of the myocardium. The systolic LV wall thickening at the lateral wall had improved significantly at five weeks (p=0.03, paired t-test). A suggestive increase in the LVEF was measured on the MRI at five weeks' study compared to the one week's study (p=0.06, paired t-test)

Diastolic function assessed by MRI

The early filling volume of the LV increased significantly in five weeks compared to one week (p=0.039, paired t-test). There was also a significant increase in the PFR (p=0.0078, paired t-test), but no change in the time to peak filling rate.

Histological evaluation of cardiomyocyte proliferation

Immunohistological staining of the von Willebrant factor revealed clusters of neovascularization especially at the scar areas and areas close to the infarction. Myocardial muscle cells stained positive for desmin. Proliferation-associated nuclear antigen Ki-67 was located in the myocytes, lymphocyte-resembling cells and, occasionally, in endothelial cells. The median number of dividing myocytes was 4.8 cells (range 0-48)/ cm2. The number of dividing myocytes was higher in the distant, non-infarcted area of the lesion (p=0.037, paired t-test). In contrast, the total cell proliferation index was lower in the distant myocardium than at the lesion area where clusters of non-myocyte cell proliferation were seen in the scar (p=0.02, paired t-test). COX-2 and macrophage marker expression was unaffected.

Study IV

Overall assessment

Mortality of all animals was 66%, 15 survived out of 44. Four animals died at the time of Ameroid application, 15 died in the ligation procedure, 1 died in the transplantation, and 9 died during the imaging procedures. Sustained ventricular fibrillation, with no response to electrical or pharmacological cardioversion was present in almost all cases. Mortality after myoblast transplantation was 2 animals out of 11 (18%). In control group, all animals survived after injection of the control medium. Assessed from the time point of treatment injections, there was no difference in mortality (p=0.51, Fisher's exact test). Median weight of the animals was 21 kg, range 18 to 26 kg, at the beginning of the study and median weight at the end of the study was 61 kg, range 50 to 73, with no differences between the groups. All animals showed ischemic changes in histology. There was increased fibrosis in all animals with no differences between the groups. In the myoblast group, myoblasts coud be found at the site of injections aligning the fibrotic area.

In vivo and histlogical evaluation of neovascularization

One week after the ligation, the pretreatment angiography in the myoblast transplantation group showed a median 100% stenosis in LCx, range 85 to 100%. At 5 weeks after the ligation, the LCx stenosis was median 100%, range 90 to 100% (p= n.s., Wilcoxon test). In the control group, the stenosis at three weeks' was median 100%, range 90% and 100%, range 90-100% at seven weeks' (p=n.s., Wilcoxon test). The degree of LCx stenosis or the size of the LCx vessels did not differ between between the groups. Filling of the LCx via collaterals according to Schwartz grade did not differ between the groups.

MRI perfusion measurements showed median slope difference 22°(range 12-64°) in the myoblast group on the pretreatment MRI and 12°(range 5-28) on post-treatment MRI (p=0.09). The median slope difference in the control group on pre-treatment MRI was 18°(range 0-42°) and on post-treatment MRI was 12°(range 0-22, p=0.12). The change in slope difference did not differ between the groups (p=0.56), indicating similar amount of ischemia between the groups in the pretreatment and post-treatment studies.

Microvessels stained by anti-vWf were seen more frequently in the myoblast group at the cell transplantation area at the lateral left ventricular wall compared to the control group (p=0.045).

Diastolic function after myoblast transplantation

MRI indicated improved diastolic filling in the myoblast group, but not in the control animals according to change in PFR between pre- and posttreatment studies. In the myoblast group, the median PFR was 279 ml/s, range 146 to 441 at one week after the ligation and median 479 ml/s, range 226 to 1012 at five weeks after the ligation (p=0.0048). The median PFR in the control animals at one week after the ligation was median 279 ml/s, range 221 to 511 and median 339 ml/s, range 267 to 606 at five weeks (p=0.18). In the myoblast transplantation group, DD increased from the pretreatment median 324 ms, range 169 to 528 into posttreatment median 535

ms, range 266 to 590 (p=0.0039). In the control group, pretreatment DD was median 322 ms, range 225 to 465 at and posttreatment median was 389 ms, range 250 to 537 (p= 0.44). There was no change in the TPR in the myoblast group, nor in the control group between the pretreatment and posttreatment studies. Heart rates were equal between the groups at pretreatment and posttreatment studies.

Systolic function after myoblast transplantation

In the myoblast group, LVEF was median 51%, range 29% to 69% at the pretreatment MRI and median 50%, range 44% to 65% in the posttreatment MRI (p=0.18). In the control group LVEF was 57%, range 42% to 62% in the pretreatment MRI and median 49%, range 41% to 65% at the posttreatment MRI (p=0.56). There was no difference between the groups. Local systolic thickening at the lateral LV wall at the LCx area, showed no improvement in either groups. In the myoblast transplanted animals, PER was 277 ml/s, range 155 to 604 in MRI at one week after the ligation and median 389 ml/s, range 211 to 655 at five weeks after the ligation (p=0.049). In the control animals PER was median 266 ml/s, range 205 to 439 at one week and median 389 ml/s, range 211-655 at five weeks after the ligation (p=0.84).

7. Discussion

Porcine Ameroid model

Porcine has many advantages in modelling acquired human heart disease. First, macroscopic anatomy of porcine heart structure resembles closely human (Crick and others 1998b). Second, coronary anatomy and individual variation in the blood supply of the LV fluctuates similarly to man (Weaver and others 1986). Third, pre-existing collateral vessels are similarly sparse in both species (Maxwell, Hearse, Yellon 1987). Fourth, the size of porcine makes possible to utilize examination methods developed for humans (Hughes 1986). Fifth, similarly in both species myocardium primarily uses nonesterified fatty acids as the preferred substrate under normal conditions, whereas during myocardial ischemia, the oxidation of fatty acids diminishes concomitant with increased glucose metabolism (Abdel-Aleem and others 1999).

Ameroid constrictors have been widely used to produce slowly progressing coronary artery stenosis in animal models of myocardial ischemia (Hughes and others 2003). When Ameroid constrictors were immersed in physiologic saline, rapid expansion during the first two weeks was followed by a slow progress (Berman and others 1956). Anyhow, in vivo changes in the physiological environment and material might affect the ability of the constrictor to absorb water (Adin and others 2004), which is the prerequisite for the expansion. Also, variations of coronary vessel distribution and size might lead to inadequate and inaccurate vessel occlusion or unpredictable myocardial lesion. Large myocardial infarction after fast occlusion of the Ameroid should be avoided in the experimental setting for myocardial ischemia and angiogenesis (Unger 2001). However, in studies of heart failure, myocardial loss and scarring is required for sufficient LV dysfunction, to show the hypothetical treatment efficacy

In our first series (study I) we showed variability in the Ameroid occlusion rate similar to previous reports (Litvak, Siderides, Vineberg 1957; Nakai and others 2005; Sereda and Adin 2005). At three weeks after the Ameroid application the mean stenosis was only 78%. Although severely stenosed at seven weeks after the Ameroid application (mean 95%), every fourth of coronaries were still patent. The unevenness of the occlusion rate can cause a requirement for larger animal cohorts. However, in our study of intramyocardial anngiogenic gene therapy, we could show a significant increase in Ameroid-induced stenosis, and progressive stenosis provided a model similar in comparison with exertion ischemia. Control group showed also spontaneous development of significant collateral circulation. This must be kept in mind, when assessing effects of collateral formation of certain treatment. Proper control group and sufficient amount of animals is a perquisite for a well-conducted study. Difference between juvenile and adult, or healthy and diseased myocardium to produce collateral vessels might also elucidate part of the spontaneous growth of the collateral vessels in animal studies, when compared to the human studies (Gaffney and others 2007).

Modification of the Ameroid model

We chose to generate more consistent coronary artery occlusion than the standard Ameroid model. For simplicity, non-absorbable suture applied during the primary surgery was used to occlude the Ameroid-narrowed coronary artery. Time point of ligation was chosen by the knowledge of already formed collaterals at three weeks, which would protect the affected area from a massive infarct. A rather uniform occlusion was produced, with significant improvement of the occlusion rate compared to our previous series of Ameroid occlusion without the ligation. The ligation process caused an infarct at the LCx perfusion area with relatively consistent lesions.

We studied the natural healing of the new model. Our main findings were, that perfusion at the border area improved, scar size diminished and local and global function of the LV improved in both systolic and diastolic measures. The findings we were able to show in a large scale of examination methods. We used cardiac MRI, SPECT, PET and angiography. All the modalities showed congruent results.

The most salient findings include scar diminishment, which is of significant importance if this model is to be used in a model of cellular transplantation after myocardial infarct. However, similar results have previously been introduced in both animal models as well as in humans (Holmes and others 1994; Holmes, Nunez, Covell 1997; Hombach and others 2005). The amount of the scar was measured by late enhancement images of MRI. Scar shrinkage is probably part of the healing process as seen in clinical cases. There is also possibility of inaccurate delineation of the scar due to odema at the early stages of infarcted myocardium.

The functional improvement of the LV is most evidently explained by improved perfusion and reversal of hibernation and stunning (Fallavollita and Canty 1999; Fallavollita, Logue, Canty 2001) Hibernating myocardium and stunning to co-exist (Hughes and others 2001b) in the Ameroid swine model. In our study, there was no mean rest ischemia according to SPECT, which suggests that the myocardium is globally stunned, although viable myocardial regions with chronically impaired resting function may have normal or reduced myocardial blood flow at rest. There was variation in the FDG uptake between individual animals. Because the animals developed variably both ischemia and infarction in the region of interest, the 18-FDG-PET deposition varied. The imaging method allowed acquisition of only one image of full thickness LV wall, and the transmural variations of 18-FDG uptake could not be spatially separated. Thus, the image is the sum of subendocardial, midmyocardial and subepicardial glucose metabolism. There are known differences in the 18-FDG uptake between these regions. While 18-FDG uptake is reduced in the infarcted myocardium and increased in the ischemic myocardium, the summation effect on the 18-FDG uptake might be neutral. Nevertheless, all animals had less deranged 18-FDG-uptake five weeks versus one week after ligation, a finding that is consistent with improved perfusion and a reduction in the amount of nonviable myocardium. Although both scar and ischemic myocardium were present, the finding of myocardial functional improvement showed clearly that ischemia was reversible.

We measured the amount of the myocyte nuclei in mitosis at the time of eutanasia. The mitotic myocyte nuclei were located at the remote part of the LV to the area of the ischemic myocardium. This could indicate compensatory activation of remote parts of the myocardium. So far, evidence of distant myocyte proliferation in the presence of stunning or hibernating

heart is sparse (Lim and others 1999). Compensatory activation of myocytes distal to the lesion area warrants further studies to demonstrate the functional benefit. The value of this kind result from an experimental model is strengthened by the similarity between human and porcine cardiac anatomy and CV disease progression. The number of proliferating myocyte nuclei at the time of eutanasia was rather low, which does not support a suggestion that myocyte proliferation might substantially affect LV function. Furthermore, the proliferation of the myocytes was only measured at one time point.

Our aim was to create a simple and easily reproducible myocardial model to regulate and to standardize the myocardial lesion as a modification of the Ameroid constrictor model and to assess the consequent spontaneous response of myocardial perfusion and collateral formation. Our hypothesis was that adaptation to ischemia before banding would reduce arrhythmia induced sudden death after coronary occlusion, and that timing of the coronary occlusion would result in a uniform infarction and thus, improve the usefulness of Ameroid constrictor model for preclinical studies of gene and/or cellular therapy. Staged banding of the Ameroid-stenosed vessel after initial ischemic adaptation of the myocardium seems a substantial improvement The spontaneous improvement of left ventricular ischemic lesion must be taken into consideration in this model.

Angiogenic potency of VEGF-C

This is the first large-animal angiogenic study demonstrating adenovirus-mediated VEGF-C gene transfer-induced collateral formation and the maintenance of myocardial function in progressive myocardial ischaemia. Our model of progressive stenosis provides a state, where natural healing process during increasing ischemia acts in concert with the therapeutic agent. In this setting, the inherent ability of myocardium to recuperate ischemia seems to be positively influenced by adVEGDF-C transfer. We are unable to show the mechanism of therapeutic action of the adVEGF-C, but the difference in perfusion with the control group suggests action via VEGF-C overexpression, rather than the adenoviral effect. Coronary artery disease patient have ischemic myocardium only during exercise, when patients with unstable angina are excluded. Thus similar condition to our model can be achieved clinically with exercise-induced angina.

In our study, we used SPECT data in the analysis of LV wall thickening and movement. In our SPECT, 24 images were obtained by ECG gating between one R-R-interval. Thus, a reliable functional status can be obtained, even when compared to MRI (Bax and others 2000). The increasing stenosis of the LCx caused deterioration of systolic thickening in the LacZ control group, but the VEGF-C treatment was able to reverse this deterioration. Anyhow, perfusion maps were unable to show difference between the groups. This might be due to the overall insensitivity of SPECT to detect minor differences in myocardial ischemia.

Development of collateral vessels was significant in both groups. Anyhow, in the VEGF-C group, the collateral vessels were more clustered at the area of injections, when compared to the control group. This focal effect of VEGF-C overexpression was further evidenced by histological samples, in which more microvessels were seen at the area of injections. We measured the microvessels with anti-von Willebrand factor immunostaining, which does not differentiate blood vessel endothelium from lymph vessel endothelium.

Metabolic assessment by 18-FDG-PET showed deterioration in the control group, when the treatment group improved or remained the same. Similarly to the LV wall thickening, this suggests beneficial effect of the VEGF-C gene transfer. Interestingly, when PET studies were performed three times during the study period, the highest glucose uptake was at five weeks after the Ameroid placement, thus indicating the probable time scale of the Ameroid occlusion.

Individual variation of the size of the LCx and right coronary artery is similar to man in porcine. This variation must be considered, when porcine is used as a model of collateral development. We used two independent interpreters blinded to the randomization to estimate the LCx size. In a semiquantitative analysis no differences between the groups could be found.

In a model of ischemic rabbit hidlimb, VEGF-C induced collateral formation, when administered both as recombinat protein or plasmid gene transfer (Witzenbichler and others 1998). Increased endothelial mitogenesis and migration as well as increased nitric oxide release after VEGF-C administration was shown in vitro. In the in vivo setting, angiogenesis and collateral formation was shown in the VEGF-C treated animals anatomically by angiography, physiologically by calf pressure measurement and also histologically.

In a clinical study of plasmid VEGF-C gene transplantation by intramyocardial injections for myocardial ischemia by Losordo et al. (Losordo and others 2002), similar results were observed. In this dose escalating study, 18 patients received plasmid VEGF-C by intramyocardial injections and were compared to 9 patients receiving saline. Double blind analysis of the material showed statistically significant reduction in anginal class and analysis of the SPECT and electromechanical mapping showed suggestive improvement in the VEGF-C gene transfer group. VEGF-C has been lately shown to primarily act on lymphatic tissue development and regeneration (Saaristo and others 2002), but overlapping in receptor binding with other VEGFs suggests that VEGF-C might have potency in the treatment of ischemic disorders. VEGF-C has also shown to act in a therapeutic manner in neointimal hyperplasia (Hiltunen and others 2000).

We showed collateral vessels clustering at the site of adVEGF-C therapy at 4 weeks after the injection. According to previous reports, at that time the adenoviral expression should already ceased off (Rutanen and others 2004c). Other VEGFs, such as VEGF-A and VEGF-D transfected with adenovirus has shown results similar to ours (Lee and others 2000a; Rutanen and others 2004b). This large animal study suggests the potential of VEFG-C gene transfer in the treatment of myocardial ischemia.

Myoblast transplantation in a porcine model

In our study, cardiac diastolic function improved in the animals receiving autologous myoblasts. The cells were transplanted at the area of ischemia and infarction. The improvement was shown in cardiac MRI. Our model closely mimics the course of human coronary disease on a large experimental animal setting. Our data demonstrate that transplantation of autologous myoblasts after brief expansion, effects comparable to extensive cultivation as reported for example in the Magic-trial, which showed improvement in myocardial remodelling (Menasche and others 2008b).

The basis of the improvement in diastolic properties of the myoblast-transplanted hearts remains obscure. We could find more microvessels at the myoblast injection area, which indi-

cates improved local perfusion and oxygen supply. Anyhow, macroscopic perfusion studies in cardiac MRI did not show difference compared to the control animals, nor did angiography show difference in collateral assessment. Thus the improved perfusion remains unconfirmed. In an animal study of myoblast transplantation enhancement of myocardial oxygenation was shown at the site of cell transplant compared with untreated control hearts. Skeletal myoblasts were labeled with oxygen sensing probes before further transplantation to heart (Wisel and others 2007). According to this, our methods of perfusion assessment might be insensitive to reach minor differences. Nevertheless, our methods are widely clinically used and reliably show clinically significant changes (Lauerma and others 1997).

We used modified Ameroid model to mimic diastolic dysfunction caused by coronary artery disease. The progressively stenosing coronary artery generates collateral dependent ischemic myocardium and the further ligation of the stenosed vessel results in infarction with border ischemia. The anatomical and physiological similarity of porcine and human heart enables to study the most common type of diastolic dysfunction, caused by myocardial ischemia. In our model, both the reduced myocardial blood flow, as well as the increased fibrosis leads to reduced and delayed LV filling. Other causes for relaxation disturbancy include myocardial hibernation, stunning and possible odema due to myocardial infarction.

According to Frank-Starling law, diastolic filling is a significant determinant of systolic contractility. Thus in oours study, the increase in peak ejection rate was expected as diastolic preload was increased. Based on our observations, the myoblast-transplanted hearts seemed to function more efficiently performing systolic work in a reduced time. However, it should be emphasized, that the duration of diastole, duration of systole and LV ejection rate are all determinants of the same cardiac cycle and are correlated to each other. When both the length and duration of the LV filling slope are increased, obviously the slope of the LV emptying is also increased. In contrast to the beneficial effects observed in diastolic functional parameters, no effect was evident in systolic parameters such as LV thickening at the site of myoblast transplantation or LVEF.

Myoblast transplantation in a cryoinjury model preserved static stiffness of the LV wall and increased strain as well as resting myocardial segment length was shown. These changes suggested improved compliance of the LV wall, implying that myoblasts make the myocardium more responsive in stretch than plain scar (Atkins and others 1999). In another study, bone marrow cell transplanted hearts were related with reduced collagen density and improvement of diastolic function was seen (Nagaya and others 2004). Angioblasts and mesenchymal stem cells has also been shown to reduce collagen in the LV wall after AMI (Kocher and others 2001b). We assessed the amount of collagen histologically in both groups to measure the effect of myoblast transplantation to fibrosis, but we were unable to show difference between the groups. To demonstrate the detailed mechanisms behind the improving diastolic properties, further studies are warranted. The main limitations of the study include the small number of the animals. Also, parallel use of echocardiography might have been advantageous, since echo is the clincal standard of diastolic mesurement. Longer surveillance might have provided information of the long-term effects of the myoblast transplantation.

Our study suggests, that systolic function is not the sole determinant of the advantage of myoblast transplantation. Despite the evident disappointment in the negative results in the primary endpoints of MAGIC trial, there was evidence of beneficial effects in remodelation (Menasche

and others 2008a). Similar results have been shown in nonrandomized studies with myoblast sheet technology (Sawa 2007). Myoblasts possess several advantages. The cell can be easily harvested and proliferated and thus there is no need for immunosuppression and a significant amount of cells can be generated in a relatively short time. Myoblasts keep the ability to contract by differentiating into myotubes and myofibrils. Although the lack of electric coupling is acknowledged, the cells are able to contract upon mechanical signalling. Myoblasts are resistant to ischaemia compared to the progenitor cells and despite the high cellular death reported after transplantation, methods to improve cell survival are under development. The main effect of the myoblast transplantation has been recently proposed to be explained by paracrine effects (Formigli and others 2007; Menasche 2007; Menasche 2008; Perez-Ilzarbe and others 2008).

Our study shows the importance of large animal studies, in which clinically relevant data can de gathered. We also stress out the superiority of cardiac MRI in the large-scale assessment of cardiac function. Improved spatial resolution and faster imaging techniques in cardiac MRI show a new standard in examing heart volume and function.

Clinical relevance of recent studies

These studies show promising novel methods in the treatment of hypoperfused mammalian heart. The use of biological methods to improve perfusion and function of heart would be especially interesting in patients, who are amenable to recent treatment practise. Thus, patients with end-stage coronary disease or end-stage heart failure would present the frontline of the new therapy. However, the therapeutic effect of adVEGF-C warrants further studies to evaluate the true effect in human coronary disease. The results in a healthy young animal cannot be directly transferred in human cases, which have been selected by being not amenable to the ongoing treatment methods. The use of myoblast transplantation has been tested in humans in a rather large series (Menasche 2008), where the primary end point of systolic improvement did not show any affect. Anyhow, there were other effects, which might warrant new consideration of the treatment outcome. While these new treatment modalities are under development, testing of the therapeutic efect should be done in appropriate biological models.

8. SUMMARY AND CONCLUSIONS

The present study evaluated VEGF-C over-expression angiogenic gene therapy and myoblast cell therapy in the treatment of coronary disease and heart failure. We used porcine coronary gradual stenosis and occlusion to model human coronary artery disease and its consequences. We made improvements in the standard Ameroid model and described the natural course of the new model. Intramyocardial autologous myoblasts were tested in this newly developed model.

I   Intramyocardial adenoviral VEGF-C gene transfer was carried out during progressive occlusion of LCx coronary artery. In the VEGF-C treated animals heart function remained, whereas in the adLacZ control animals the heart function deteriorated by increasing coronary stenosis. More microvessels was clustered at the injection area.

II Due to inconsistent stenosis produced by Ameroid, a new modification of the Ameroid model was developed. With this new stenosis-ligation-model, more reliable stenosis could be achieved with rather constant lateral LV wall infarction.

III The spontaneous response of the heart to the new stenosis-ligation-model was shown by MRI, SPECT, PET and angiography. A wide range of natural improvement was measured, including systolic and diastolic LV improvement, reduced infarct size according to MRI and improved perfusion confirming recuperation of hibernation and stunning at the border zone at the infarct penumbra area. Histological double-stainings were employed to measure the amount of cardiac regeneration by dividing cardiomyocytes, which indicated more cardiomyocyte mitosis at the romote area of the infarct.

IV Low dose myoblast transplantation at the area at risk of the newly developed model suggested improvement of diastolic function of LV. We were able to locate the transplanted cells 4 weeks after the transplantation. EF or local systolic thickening showed no improvement, when compared to the control group

Our study suggests potential of VEGF-C treatment in the use of progressive myocardial ischemia. VEGF-C deserves further studies as a therapeutic option in cardiovascular disease, to become a viable option as a viable angiogenic treatment in coronary disease.

Inconsistent coronary occlusion in Ameroid constrictor model might disturb the experimental setting in animal models of ischemia. By ligation of the Ameroid-stenosed proximal LCx coronary artery at three weeks after the Ameroid application, leads to consistent stenosis of the vessel as to reproducible infarction of the vessel. The natural healing process in this model must be taken into consideration when experimentally used.

Myoblast transplantation might have effect on cardiac function even when used in low dose setting. Suggestion of diastolic improvement was shown, whereas no improvement in systolic parameters was shown.

9. Acknowledgements

10. References

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